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APPLICATION

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TITLE: NOVEL LOW DENSITY LIPOPROTEIN BINDING PROTEINS AND THEIR USE IN DIAGNOSING AND TREATING ATHEROSCLEROSIS

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NOVEL LOW DENSITY LIPOPROTEIN BINDING PROTEINS AND THEIR USE IN DIAGNOSING AND TREATING ATHEROSCLEROSIS

Cross Reference to Related Applications

This application is a continuation-in-part of USSN 09/517,849, filed March 2, 2000, which was a continuation-in part of USSN 08/979,608, filed November 26, 1997, which claimed priority from USSN 60/031,930, filed November 27, 1996, and USSN 60/048,547, filed June 3, 1997.

Field of the Invention

This invention relates to novel polypeptides (LBPs) which bind to low density lipoprotein (LDL), polynucleotides which encode these polypeptides, and treatments, diagnoses and therapeutic agents for atherosclerosis.

Background of the Invention

Atherosclerosis is the principal cause of heart attacks and strokes. It has been reported that about 50% of all deaths in the United States, Europe and Japan are due to atherosclerosis. Atherosclerotic lesions in the arterial wall characterize atherosclerosis. Cholesteryl esters (CE) are present in these atherosclerotic lesions. Low density lipoprotein (LDL) has been shown to be the major carrier of plasma CE, and has been implicated as the agent by which CE enter the atherosclerotic lesions.

Scattered groups of lipid-filled macrophages, called foam cells, are the first visible signs of atherosclerosis and are described as type I lesions. These macrophages are reported to contain CE derived from LDL. The macrophages recognize oxidized LDL, but not native LDL, and become foam cells by phagocytosing oxidized LDL. Larger, more organized collections of foam cells, fatty streaks, represent type II lesions. These lesions further develop into complex lesions called plaques, which can result in impeding the flow of blood in the artery.

It is widely believed that accumulation of LDL in the artery depends on the presence of functionally modified endothelial cells in the arterial wall. It has been reported in animal models of atherosclerosis that LDL, both native LDL and methylated

LDL, accumulates focally and irreversibly only at the edges of regenerating endothelial islands in aortic lesions, where functionally modified endothelial cells are present, but not in the centers of these islands where endothelial regeneration is completed. Similarly, LDL accumulates in human atherosclerotic lesions. The mechanism by which the LDL accumulates focally and irreversibly in arterial lesions has not heretofore been understood.

Summary of the Invention

It is an object of the invention to provide polypeptides which bind to LDL.

It is yet another object of the invention to provide a method for determining if an animal is at risk for atherosclerosis.

It is yet another object of the invention to provide a method for evaluating an agent for use in treating atherosclerosis. It is yet another object of the invention to provide a method for treating atherosclerosis.

Still another object of the invention is to utilize an LBP (low density lipoprotein binding protein) gene and/or polypeptide, or fragments, analogs and variants thereof, to aid in the treatment, diagnosis and/or identification of therapeutic agents for atherosclerosis.

In one aspect, the invention features an isolated polynucleotide comprising a polynucleotide encoding the polypeptide comprising the amino acid sequence as set forth in SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8; SEQ ID NO: 9; SEQ ID NO:43; SEQ ID NO:44; SEQ ID NO:47 or a polynucleotide capable of hybridizing to and which is at least about 95% identical to any of the above polynucleotides and wherein the encoded polypeptide is capable of binding to LDL; or a biologically active fragment of any of the above polynucleotides wherein the encoded polypeptide is capable of binding to LDL.

In certain embodiments, the polynucleotide comprises the nucleic acid sequence as set forth in SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17; SEQ ID NO: 18; SEQ ID NO:45; SEQ ID NO:46; SEQ ID NO:48.

Another aspect of the invention is an isolated polypeptide comprising a

polypeptide having the amino acid sequence as set forth in SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8; SEQ ID NO: 9; SEQ ID NO: 43; SEQ ID NO:44; SEQ ID NO:47; or a polypeptide which is at least about 95% identical to any of the above polypeptides and wherein the polypeptide is capable of binding to LDL; or a biologically active fragment of any of the above polypeptides wherein the fragment is capable of binding to LDL.

Another aspect of the invention is a method for determining if an animal is at risk for atherosclerosis. An animal is provided. An aspect of LBP metabolism or structure is evaluated in the animal. An abnormality in the aspect of LBP metabolism or structure is diagnostic of being at risk for atherosclerosis. Another aspect of the invention is a method for evaluating an agent for use in treating atherosclerosis. A test cell, cell-free system or animal is provided. An agent is provided. The agent is administered to the test cell, cell-free system or animal in a therapeutically effective amount. The effect of the agent on an aspect of LBP metabolism or structure is evaluated. A change in the aspect of LBP metabolism or structure is indicative of the usefulness of the agent in treating atherosclerosis.

Another aspect of the invention is a method for evaluating an agent for the ability to alter the binding of LBP polypeptide to a binding molecule, e.g., native LDL, modified LDL, e.g., methylated LDL or oxidized LDL, or an arterial extracellular matrix structural component. An agent is provided. An LBP polypeptide is provided. A binding molecule is provided. The agent, LBP polypeptide and binding molecule are combined. The formation of a complex comprising the LBP polypeptide and binding molecule is detected. An alteration in the formation of the complex in the presence of the agent as compared to in the absence of the agent is indicative of the agent altering the binding of the LBP polypeptide to the binding molecule.

Another aspect of the invention is a method for evaluating an agent for the ability to bind to an LBP polypeptide. An agent is provided. An LBP polypeptide is provided. The agent is contacted with the LBP polypeptide. The ability of the agent to bind to the LBP polypeptide is evaluated.

Another aspect of the invention is a method for evaluating an agent for the ability to bind to a nucleic acid encoding an LBP regulatory sequence. An agent is provided. A

nucleic acid encoding an LBP regulatory sequence is provided. The agent is contacted with the nucleic acid. The ability of the agent to bind to the nucleic acid is evaluated.

Another aspect of the invention is a method for treating atherosclerosis in an animal. An animal in need of treatment for atherosclerosis is provided. An agent capable of altering an aspect of LBP structure or metabolism is provided. The agent is administered to the animal in a therapeutically effective amount such that treatment of the atherosclerosis occurs. In certain embodiments, the agent is an LBP polypeptide, e.g., LBP-1, LBP-2 or LBP-3, or a biologically active fragment or analog thereof. In certain embodiments, the agent is a polypeptide of no more than about 100, 50, 30, 20, 10, 5, 4, 3 or 2 amino acid residues in length. In certain embodiments, the agent is a polypeptide having an amino acid sequence that includes at least about 20%, 40%, 60%, 80%, 90%, 95% or 98% acidic amino acid residues.

Another aspect of the invention is a method for treating an animal at risk for atherosclerosis. An animal at risk for atherosclerosis is provided. An agent capable of altering an aspect of LBP structure or metabolism is provided. The agent is administered to the animal in a therapeutically effective amount such that treatment of the animal occurs.

Another aspect of the invention is a method for treating a cell having an abnormality in structure or metabolism of LBP. A cell having an abnormality in structure or metabolism of LBP is provided. An agent capable of altering an aspect of LBP structure or metabolism is provided. The agent is administered to the cell in a therapeutically effective amount such that treatment of the cell occurs.

Another aspect of the invention is a pharmaceutical composition for treating atherosclerosis in an animal comprising a therapeutically effective amount of an agent, the agent being capable of altering an aspect of LBP metabolism or structure in the animal so as to result in treatment of the atherosclerosis, and a pharmaceutically acceptable carrier.

Another aspect of the invention is a vaccine composition for treating atherosclerosis in an animal comprising a therapeutically effective amount of an agent, the agent being capable of altering an aspect of LBP metabolism or structure in the animal so as to result in treatment of the atherosclerosis, and a pharmaceutically

acceptable carrier.

Another aspect of the invention is a method for diagnosing atherosclerotic lesions in an animal. An animal is provided. A labeled agent capable of binding to LBP, e.g., LBP-1, LBP-2 or LBP-3, present in atherosclerotic lesions is provided. The labeled agent is administered to the animal under conditions which allow the labeled agent to interact with the LBP so as to result in labeled LBP. The localization or quantification of the labeled LBP is determined by imaging so as to diagnose the presence of atherosclerotic lesions in the animal.

Another aspect of the invention is a method for immunizing an animal against an LBP, e.g., LBP-1, LBP-2 or LBP-3, or fragment or analog thereof. An animal having LDL is provided. The LBP or fragment or analog thereof is administered to the animal so as to stimulate antibody production by the animal to the LBP or fragment or analog thereof such that binding of the LBP to the LDL is altered, e.g., decreased or increased.

Another aspect of the invention is a method of making a fragment or analog of LBP polypeptide, the fragment or analog having the ability to bind to native LDL and to modified LDL, e.g., methylated LDL, oxidized LDL, acetylated LDL, or cyclohexanedione-treated LDL. An LBP polypeptide is provided. The sequence of the LBP polypeptide is altered. The altered LBP polypeptide is tested for the ability to bind to modified LDL and native LDL.

Yet another aspect of the invention is a method for isolating a cDNA encoding an LBP. A cDNA library is provided. The cDNA library is screened for a cDNA encoding a polypeptide which binds to native LDL and modified LDL, e.g., methylated LDL or oxidized LDL. The cDNA which encodes the polypeptide is isolated, the cDNA encoding an LBP.

The above and other features, objects and advantages of the present invention will be better understood by a reading of the following specification in conjunction with the drawings.

Brief Description of the Drawings

Fig. 1 depicts the amino acid sequence of rabbit LBP-1 (SEQ ID NO: 1). Differences in amino acids between rabbit and human LBP-1 are depicted in bold type.

Fig. 2A depicts the nucleotide sequence (SEQ ID NO: 48) and amino acid sequence (SEQ ID NO: 47) of rabbit LBP-2.

Fig. 2B depicts a portion of the amino acid sequence of rabbit LBP-2 (SEQ ID NO: 2). Differences in amino acids between rabbit and human LBP-2 are depicted in bold type. Where the sequences depicted in Fig. 2A and Fig. 2B differ, Fig. 2A represents the rabbit LBP-2 sequence.

Fig. 3 depicts the amino acid sequence of amino acids 319 to 350 of rabbit LBP-2 (SEQ ID NO: 3).

Fig. 4 depicts the amino acid sequence of amino acids 299 to 350 of rabbit LBP-2 (SEQ ID No: 4).

Fig. 5 depicts the amino acid sequence of rabbit LBP-3 (SEQ ID NO: 5). Differences in amino acids between rabbit and human LBP-3 are depicted in bold type.

Fig. 6 depicts the amino acid sequence of human LBP-1 (SEQ ID NO: 6). Differences in amino acids between rabbit and human LBP-1 are depicted in bold type.

Fig. 7A depicts the nucleotide sequence (SEQ ID NO: 45) and amino acid sequence (SEQ ID NO: 43) of human LBP-2.

Fig. 7B depicts the amino acid sequence of amino acids 322 to 538 of human LBP-2 (SEQ ID NO: 7). Differences in amino acids between rabbit and human LBP-2 are depicted in bold type.

Fig. 8A depicts the nucleotide sequence (SEQ ID NO: 46) and amino acid sequence (SEQ ID NO: 44) of human LBP-3.

Fig. 8B depicts the amino acid sequence of amino acids 17 to 546 of human LBP-3 (SEQ ID NO: 8). Differences in amino acids between rabbit and human LBP-3 are depicted in bold type. Where the sequences depicted in Fig. 8A and Fig. 8B differ, Fig. 8A represents the human LBP-3 sequence.

Fig. 9 depicts the amino acid sequence of amino acids 14 to 33 of human or rabbit LBP-1, called BHF-1 (SEQ ID NO: 9).

Fig. 10 depicts the cDNA sequence encoding rabbit LBP-1 (SEQ ID NO: 10) and the corresponding amino acid sequence. Differences in amino acids between rabbit and human LBP-1 are depicted in bold type.

Fig. 11 depicts a cDNA sequence encoding a portion of rabbit LBP-2 (SEQ ID

NO: 11) and the corresponding amino acid sequence. Differences in amino acids between rabbit and human LBP-2 are depicted in bold type. Where the sequences depicted in Fig. 2A and Fig. 11 differ, Fig. 2A represents the rabbit LBP-2 sequence.

Fig. 12 depicts a cDNA sequence of nucleotides 256 to 1617 (SEQ ID NO: 12) of SEQ ID NO: 11 of rabbit LBP-2 and the corresponding amino acid sequence.

Fig. 13 depicts a cDNA sequence of nucleotides 196 to 1617 (SEQ ID NO: 13) of SEQ ID NO: 11 of rabbit LBP-2 and the corresponding amino acid sequence.

Fig. 14 depicts the cDNA sequence encoding rabbit LBP-3 (SEQ ID NO: 14) and the corresponding amino acid sequence. Differences in amino acids between rabbit and human LBP-3 are depicted in bold type.

Fig. 15 depicts the cDNA sequence encoding human LBP-1 (SEQ ID NO: 15) and the corresponding amino acid sequence. Differences in amino acids between rabbit and human LBP-1 are depicted in bold type.

Fig. 16 depicts a cDNA sequence encoding a portion of human LBP-2 (SEQ ID NO: 16) and the corresponding amino acid sequence. Differences in amino acids between rabbit and human LBP-2 are depicted in bold type.

Fig. 17 depicts a cDNA sequence encoding a portion of human LBP-3 (SEQ ID NO: 17) and the corresponding amino acid sequence. Differences in amino acids between rabbit and human LBP-3 are depicted in bold type. Where the sequences depicted in Fig. 8A and Fig. 17 differ, Fig. 8A represents the human LBP-3 sequence.

Fig. 18 depicts the cDNA sequence encoding BHF-1 (SEQ ID NO: 18).

Fig. 19 corresponds to the amino acid sequence of rabbit LBP-1 (top sequence) in alignment with the amino acid sequence of human LBP-1 (bottom sequence).

Fig. 20 corresponds to the amino acid sequence of a portion of the amino acid sequence of rabbit LBP-2 (top sequence) in alignment with a portion of the amino acid sequence of human LBP-2 (bottom sequence).

Fig. 21 corresponds to the amino acid sequence of rabbit LBP-3 (top sequence) in alignment with the amino acid sequence of a portion of human LBP-3 (bottom sequence).

Fig. 22 depicts the genomic sequence of human LBP-1.

Fig. 23 depicts the genomic sequence of human LBP-2.

Fig. 24 depicts the genomic sequence of human LBP-3.

Detailed Description

In accordance with aspects of the present invention, there are provided novel mature human and rabbit polypeptides, LBP-1, LBP-2 and LBP-3, and biologically active analogs and fragments thereof, and there are provided isolated polynucleotides which encode such polypeptides. LBP is an abbreviation for low density lipoprotein (LDL) binding protein. The terms polynucleotide, nucleotide and oligonucleotide are used interchangeably herein, and the terms polypeptides, proteins and peptides are used interchangeably herein.

This invention provides for an isolated polynucleotide comprising a polynucleotide encoding the polypeptide having the amino acid sequence of rabbit LBP-1 as set forth in Fig. 1 (SEQ ID NO: l); rabbit LBP-2 as set forth in Fig. 2A (SEQ ID NO: 47); a portion of rabbit LBP-2 as set forth in Fig. 2B (SEQ ID NO: 2); 319 to 350 of rabbit LBP-2 as set forth in Fig. 3 (SEQ ID NO: 3); 299 to 350 of rabbit LBP-2 as set forth in Fig. 4 (SEQ ID NO: 4); rabbit LBP-3 as set forth in Fig. 5 (SEQ ID NO: 5); human LBP-1 as set forth in Fig. 6 (SEQ ID NO: 6); human LBP-2 as set forth in Fig. 7A (SEQ ID NO: 43); 322 to 538 of human LBP-2 as set forth in Fig. 7B (SEQ ID NO: 7); human LBP-3 as set forth in Fig. 8A (SEQ ID NO: 44); 17-546 of human LBP-3 as set forth in Fig. 8B (SEQ ID NO: 8); 14 to 33 of human or rabbit LBP-1, called BHF-1, as set forth in Fig. 9 (SEQ ID NO: 9); a polynucleotide capable of hybridizing to and which is at least about 80% identical, more preferably at least about 90% identical, more preferably yet at least about 95% identical, and most preferably at least about 98% identical to any of the above polynucleotides, and wherein the encoded polypeptide is capable of binding to LDL; or a biologically active fragment of any of the above polynucleotides wherein the encoded polypeptide is capable of binding to LDL.

This invention also includes an isolated polynucleotide comprising a polynucleotide encoding the polypeptide having amino acid residues 329-343 (SEQ ID NO: 19), 329-354 (SEQ ID NO: 20), 344-354 (SEQ ID NO: 21) or 529-538 (SEQ ID NO: 22) of human LBP-2 as set forth in Fig. 7A (SEQ ID NO: 43); amino acid residues 14-43 (SEQ ID NO: 23) or 38-43 (SEQ ID NO: 24) of rabbit or human LBP-1 as set forth

in Fig. 1 (SEQ ID NO: 1) and Fig. 6 (SEQ ID NO: 6); amino acid residues 338-353 (SEQ ID NO: 25), 338-365 (SEQ ID NO: 26), 354-365 (SEQ ID NO: 27) or 444-453 (SEQ ID NO: 28) of rabbit LBP-2 as set forth in Fig. 2A (SEQ ID NO: 47); amino acid residues 96-110 (SEQ ID NO: 29) of rabbit LBP-3 as set forth in Fig. 5 (SEQ ID NO: 5); amino acid residues 69-75 (SEQ ID NO: 41) of human LBP-3 as set forth in Fig. 8A (SEQ ID NO: 44); a polynucleotide capable of hybridizing to and which is at least about 80% identical, more preferably at least about 90% identical, more preferably yet at least about 95% identical, and most preferably at least about 98% identical to any of the above polynucleotides, and wherein the encoded polypeptide is capable of binding to LDL; or a biologically active fragment of any of the above polynucleotides wherein the encoded polypeptide is capable of binding to LDL.

By a polynucleotide encoding a polypeptide is meant a polynucleotide which includes only coding sequence for the polypeptide, as well as a polynucleotide which includes additional coding and/or non-coding sequences. Thus, e.g., the polynucleotides which encode for the mature polypeptides of Figs. 1-9 (SEQ ID NOS: 1-9, 43, 44 and 47) may include only the coding sequence for the mature polypeptide; the coding sequence for the mature polypeptide and additional coding sequence such as a leader or secretory sequence or a proprotein sequence; the coding sequence for the mature polypeptide (and optionally additional coding sequence) and non-coding sequence, such as introns or non-coding sequences 5' and/or 3' of the coding sequence for the mature polypeptide. The polynucleotides of the invention are also meant to include polynucleotides in which the coding sequence for the mature polypeptide is fused in the same reading frame to a polynucleotide sequence which aids in expression and/or secretion of a polypeptide from a host cell, e.g., a leader sequence. The polynucleotides are also meant to include polynucleotides in which the coding sequence is fused in frame to a marker sequence which, e.g., allows for purification of the polypeptide.

The polynucleotides of the present invention may be in the form of RNA, DNA or PNA, e.g., cRNA, cDNA, genomic DNA, or synthetic DNA, RNA or PNA. The DNA may be double-stranded or single stranded, and if single stranded may be the coding strand or non-coding (anti-sense) strand.

In preferred embodiments, the polynucleotide comprises the nucleic acid of

rabbit LBP-1 as set forth in Fig. 10 (SEQ ID NO: 10); rabbit LBP-2 as set forth in Fig. 2A (SEQ ID NO:48) or Fig. 11 (SEQ ID NO:11); nucleotide 256 to 1617 of SEQ ID NO: 11 of rabbit LBP-2 as set forth in Fig. 12 (SEQ ID NO: 12); nucleotide 196 to 1617 of SEQ ID NO: 11 of rabbit LBP-2 as set forth in Fig. 13 (SEQ ID NO: 13); rabbit LBP-3 as set forth in Fig. 14 (SEQ ID NO: 14); human LBP-1 as set forth in Fig. 15 (SEQ ID NO: 15); human LBP-2 as set forth in Fig. 7A (SEQ ID NO: 45) or Fig. 16 (SEQ ID NO: 16); human LBP-3 as set forth in Fig. 8A (SEQ ID NO: 46) or Fig. 17 (SEQ ID NO: 17); or nucleotide 97 to 156 of rabbit LBP-1 or nucleotide 157 to 216 of human LBP-1, (BHF-l), as set forth in Fig. 18 (SEQ ID NO: 18).

In other preferred embodiments, the polynucleotide comprises the nucleic acid as set forth in SEQ ID NO:30 (GAAGAGGAAGAAGATGATGATGAAGATGAAGATGAAGAAGATGAT), SEQ ID NO:31 (GAAGAGGAAGAAGATGATGATGAAGATGAAGATGAAGAAGA TGAT GTGTCAGAGGGCTCTGAAGTGCCCGAGAGTGAC), SEQ ID NO:32 (GTGTCAGAGGGCTCTGAAGTGCCCGAGAGTGAC), SEQ ID NO:33 (GAGGATGATGACCCCGATGGCTTCTTAGGC), SEQ ID NO:34 (GTGGACGTGGATGAATATGACGAGAACAAGTTCGTGGACGAA GAAGATGGGGGCGACGGCCAGGCCGGGCCCGACGAGGGCGAGGTGGAC), SEQ ID NO:35 (GACGAGGGCGAGGTGGAC), SEQ ID NO:36 (GAGGAGGAGGAGGAGGAGGAGGAAGACGACGAGGACGACG ACGACGAC), SEQ ID NO:37 (GAGGAGGAGGAGGAGGAGGAGGAAGACGACGAGGACGACG ACGACGACGTCGTGTCCGAGGGCTCGGAGGTGCCCGAGAGCGAT), SEQ ID NO:38 (GTCGTGTCCGAGGGCTCGGAGGTGCCCGAGAGCGAT), SEQ ID NO:39 (CCCCCCGGGAAGCCAGCCCTCCCAGGAGCC), SEQ ID NO:40 (GAGGATGGGGTCCAGGGTGAGCCCCCTGAACCTGAAGATGCA GAG), or SEQ ID NO:42 (CGTGATGTCTCTGAGGAGCTG).

The coding sequence which encodes the mature polypeptide may be identical to the coding sequences shown in Figs. 2A, 7A, 8A and 10-18 (SEQ ID NOS: 10-18, 45, 46, and 48) or SEQ ID NOS: 30-40 or 42, or may be a different coding sequence which coding sequence, as a result of the redundancy or degeneracy of the genetic code,

encodes the same mature polypeptides as the DNA of Figs. 2A, 7A, 8A and 10-18 (SEQ ID NOS: 10-18, 45, 46, and 48) and SEQ ID NOS: 30-40 and 42.

This invention aiso includes recombinant vectors comprising the polynucleotides described above. The vector can be, e.g., a plasmid, a viral particle or a phage. In certain embodiments, the recombinant vector is an expression vector. The vectors may also include various marker genes which are useful in identifying cells containing such vectors.

This invention also includes a cell comprising such a recombinant vector. The recombinant vectors described herein can be introduced into a host cell, e.g., by transformation, transfection or infection.

This invention also includes a method for producing an LBP comprising culturing such a cell under conditions that permit expression of the LBP.

This invention also includes an isolated polypeptide comprising a polypeptide having the amino acid sequence as set forth in Fig. 1 (SEQ ID NO: 1); Fig. 2A (SEQ ID NO: 47); Fig. 2B (SEQ ID NO: 2); Fig. 3 (SEQ ID NO: 3); Fig. 4 (SEQ ID NO: 4); Fig. 5 (SEQ ID NO: 5); Fig. 6 (SEQ ID NO: 6); Fig. 7A (SEQ ID NO: 43); Fig. 7B (SEQ ID No: 7); Fig. 8A (SEQ ID NO: 44); Fig. 8B (SEQ ID NO: 8); or Fig. 9 (SEQ ID NO: 9); or a polypeptide which is at least about 80% identical, more preferably at least about 90% identical, more preferably yet at least about 95% identical, and most preferably at least about 98% identical to the above polypeptides, and wherein said polypeptide is capable of binding to LDL; or a biologically active fragment of any of the above polypeptides wherein the fragment is capable of binding to LDL. Differences in amino acids between the rabbit and human LBP-1, LBP-2 and LBP-3 genes are depicted in bold type in the figures. Differences in the amino acid sequences between rabbit and human LBP-1, LBP-2 and LBP-3 are also specifically shown in Figs. 19, 20 and 21, respectively.

This invention also includes an isolated polypeptide comprising a polypeptide having amino acid residues 329-343 (SEQ ID NO: 19), 329-354 (SEQ ID NO: 20), 344-354 (SEQ ID NO: 21) or 529-538 (SEQ ID NO: 22) as set forth in Fig. 7A (SEQ ID NO: 47); amino acid residues 14-43 (SEQ ID NO: 23) or 38-43 (SEQ ID NO: 24) as set forth in Fig. 1 (SEQ ID NO: 1) and Fig. 6 (SEQ ID NO: 6); amino acid residues 338-353 (SEQ ID NO: 25), 338-365 (SEQ ID NO: 26), 354-365 (SEQ ID NO: 27) or 444-453 (SEQ ID

NO: 28) as set forth in Fig. 2A (SEQ ID NO: 47); amino acid residues 96-110 (SEQ ID NO: 29) as set forth in Fig. 5 (SEQ ID NO: 5); and amino acid residues 69-75 (SEQ ID NO: 41) as set forth in Fig. 8A (SEQ ID NO: 8); or a polypeptide which is at least about 80%identical, more preferably at least about 90% identical, more preferably yet at least about 95% identical, and most preferably at least about 98% identical to the above polypeptides, and wherein said polypeptide is capable of binding to LDL; or a biologically active fragment of any of the above polypeptides wherein the fragment is capable of binding to LDL.

The polypeptides of the invention are meant to include, e.g., a naturally purified product, a chemically synthesized product, and a recombinantly derived product.

The polypeptides can be used, e.g., to bind to LDL, thereby inhibiting formation of atherosclerotic plaques. The polypeptides can also be used, e.g., in gene therapy, by expression of such polypeptides in vivo. The polypeptides can also be used in pharmaceutical or vaccine compositions. The polypeptides can also be used as immunogens to produce antibodies thereto, which in turn, can be used as antagonists to the LBP polypeptides.

Without being bound by any theory, it is believed that the LBPs provide the mechanism by which atherosclerosis is promoted through LDL oxidation. The LBPs are believed to be required in order for focal, irreversible LDL binding to occur at the arterial wall, and that such binding is a critical early event in atherosclerosis because it allows the time necessary for LDL to be changed from its native state to a fully oxidized state.

Since oxidized, but not native, LDL is a foreign protein, macrophages ingest it, first becoming the foam cells of type I lesions, and subsequently forming the fatty streaks of type II lesions.

This invention also includes a method for determining if an animal is at risk for atherosclerosis. An animal is provided. An aspect of LBP metabolism or structure is evaluated in the animal. An abnormality in the aspect of LBP metabolism or structure is diagnostic of being at risk for atherosclerosis.

By atherosclerosis is meant a disease or condition which comprises several stages which blend imperceptibly into each other, including irreversible binding of LDL, LDL oxidation, macrophage recruitment, blockage of the artery and tissue death

(infarction).

By animal is meant human as well as non-human animals. Nonhuman animals include, e.g., mammals, birds, reptiles, amphibians, fish, insects and protozoa. Preferably, the nonhuman animal is a mammal, e.g., a rabbit, a rodent, e.g., a mouse, rat or guinea pig, a primate, e.g., a monkey, or a pig. An animal also includes transgenic non-human animals. The term transgenic animal is meant to include an animal that has gained new genetic information from the introduction of foreign DNA, i.e., partly or entirely heterologous DNA, into the DNA of its cells; or introduction of a lesion, e.g., an, in vitro induced mutation, e.g., a deletion or other chromosomal rearrangement into the DNA of its cells; or introduction of homologous DNA into the DNA of its cells in such a way as to alter the genome of the cell into which the DNA is inserted, e.g., it is inserted at a location which differs from that of the natural gene or its insertion results in a knockout or replacement of the homologous host gene or results in altered and/or regulatable expression and/or metabolism of the gene. The animal may include a transgene in all of its cells including germ line cells, or in only one or some of its cells. Transgenic animals of the invention can serve as a model for studying atherosclerosis or for evaluating agents to treat atherosclerosis.

In certain embodiments, the determination for being at risk for atherosclerosis is done in a prenatal animal.

By LBP is meant a low density lipoprotein (LDL) binding protein which is capable of binding LDL and methylated LDL. By methylated LDL is meant that about 50% to about 90% of the lysine residues of LDL have a methyl group chemically attached. Methylated LDL is not recognized by previously reported cell surface receptors. See, e.g., Weisgraber et al., J. Biol. Chem. 253: 9053-9062 (1978). In certain embodiments, the LBP is also capable of binding oxidized LDL. In certain preferred embodiments, the binding of LDL to an LBP is irreversible. In certain preferred embodiments, the LBP does not transport the LDL to any intracellular compartment. Examples of LBPs are LBP-1, LBP-2 and LBP-3 described herein.

By LBP metabolism is meant any aspect of the production, release, expression, function, action, interaction or regulation of LBP. The metabolism of LBP includes modifications, e.g., covalent or non-covalent modifications, of LBP polypeptide. The

metabolism of LBP includes modifications, e.g., covalent or noncovalent modifications, that LBP induces in other substances.

The metabolism of LBP also includes changes in the distribution of LBP polypeptide, and changes LBP induces in the distribution of other substances.

Any aspect of LBP metabolism can be evaluated. The methods used are standard techniques known to those skilled in the art and can be found in standard references, e.g., Auaubel et al., ed., Current Protocols in Mol. Biology, New York: John Wiley & Sons, 1990; Kriegler, M., ed., Gene Transfer and Expression, Stockton Press, New York, NY, 1989; pDisplay gene expression system (Invitrogen, Carlsbad, CA). Preferred examples of LBP metabolism that can be evaluated include the binding activity of LBP polypeptide to a binding molecule, e.g., LDL; the transactivation activity of LBP polypeptide on a target gene; the level of LBP protein; the level of LBP mRNA; the level of LBP modifications, e.g., phosphorylation, glycosylation or acylation; or the effect of LBP expression on transfected mammalian cell binding of LDL.

By binding molecule is meant any molecule to which LBP can bind, e.g., a nucleic acid, e.g., a DNA regulatory region, a protein, e.g., LDL, a metabolite, a peptide mimetic, a non-peptide mimetic, an antibody, or any other type of ligand. In certain preferred embodiments, the aspect of LBP metabolism that is evaluated is the ability of LBP to bind to native LDL and/or methylated LDL and/or oxidized LDL. Binding to LDL can be shown, e.g., by antibodies against LDL, affinity chromatography, affinity coelectrophoresis (ACE) assays, or ELISA assays. See Examples. In other embodiments, it is the ability of LBP to bind to an arterial extracellular matrix structural component that is evaluated. Examples of such components include proteoglycans, e.g., chondroitin sulfate proteoglycans and heparin sulfate proteoglycans; elastin; collagen; fibronectin; vitronectin; integrins; and related extracellular matrix molecules. Binding to arterial extracellular matrix structural components can be shown by standard methods known to those skilled in the art, e.g., by ELISA assays. Primary antibodies to the LBP are then added, followed by an enzyme-conjugated secondary antibody to the primary antibody, which produces a stable color in the presence of an appropriate substrate, and color development on the plates is measured in a microtiter plate reader.

Transactivation of a target gene by LBP can be determined, e.g., in a transient

transfection assay in which the promoter of the target gene is linked to a reporter gene, e.g., β-galactosidase or luciferase, and co-transfected with an LBP expression vector. Such evaluations can be done in vitro or in vivo. Levels of LBP protein, mRNA or phosphorylation, can be measured, e.g., in a sample, e.g., a tissue sample, e.g., arterial wall, by standard methods known to those skilled in the art.

In certain embodiments, an aspect of LBP structure is evaluated, e.g., LBP gene structure or LBP protein structure. For example, primary, secondary or tertiary structures can be evaluated. For example, the DNA sequence of the gene is determined and/or the amino acid sequence of the protein is determined. Standard cloning and sequencing methods can be used as are known to those skilled in the art. In certain embodiments, the binding activity of an antisense nucleic acid with the cellular LBP mRNA and/or genomic DNA is determined using standard methods known to those skilled in the art so as to detect the presence or absence of the target mRNA or DNA sequences to which the antisense nucleic acid would normally specifically bind.

The risk for atherosclerosis that is determined can be a reduced risk or an increased risk as compared to a normal animal. For example, an abnormality which would give a reduced risk is an inactive LBP polypeptide. An abnormality which would give an increased risk would be, e.g., an LBP polypeptide that has higher activity, e.g., LDL binding activity, than native LBP polypeptide.

The invention also includes a method for evaluating an agent for use in treating atherosclerosis. A test cell, cell-free system or animal is provided. An agent is provided. The agent is administered to the test cell, cell-free system or animal in a therapeutically effective amount. The effect of the agent on an aspect of LBP metabolism or structure is evaluated. A change in the aspect of LBP metabolism or structure is indicative of the usefulness of the agent in treating atherosclerosis.

In certain embodiments, the method employs two phases for evaluating an agent for use in treating atherosclerosis, an initial in vitro phase and then an in vivo phase. The agent is administered to the test cell or cell-free system in vitro, and if a change in an aspect of LBP metabolism occurs, then the agent is further administered to a test animal in a therapeutically effective amount and evaluated in vivo for an effect of the agent on an aspect of LBP metabolism.

By cell is meant a cell or a group of cells, or a cell that is part of an animal. The cell can be a human or non-human cell. Cell is also meant to include a transgenic cell. The cell can be obtained, e.g., from a culture or from an animal. Animals are meant to include, e.g., natural animals and non-human transgenic animals. In certain embodiments, the transgenic cell or nonhuman transgenic animal has an LBP transgene, or fragment or analog thereof. In certain embodiments, the transgenic cell or non-human transgenic animal has a knockout for the LBP gene.

The test cell, cell-free system or animal can have a wild type pattern or a non-wild type pattern of LBP metabolism. A non-wild type pattern of LBP metabolism can result, e.g., from under-expression, over-expression, no expression, or a temporal, site or distribution change. Such a non-wild type pattern can result, e.g., from one or more mutations in the LBP gene, in a binding molecule gene, a regulatory gene, or in any other gene which directly or indirectly affects LBP metabolism. A mutation is meant to include, e.g., an alteration, e.g., in gross or fine structure, in a nucleic acid. Examples include single base pair alterations, e.g., missense or nonsense mutations, frameshifts, deletions, insertions and translocations. Mutations can be dominant or recessive. Mutations can be homozygous or heterozygous. Preferably, an aspect of LBP-1, LBP-2 or LBP-3 metabolism is evaluated.

An agent is meant to include, e.g., any substance, e.g., an anti-atherosclerosis drug. The agent of this invention preferably can change an aspect of LBP metabolism. Such change can be the result of any of a variety of events, including, e.g., preventing or reducing interaction between LBP and a binding molecule, e.g., LDL or an arterial extracellular matrix structural component; inactivating LBP and/or the binding molecule, e.g., by cleavage or other modification; altering the affinity of LBP and the binding molecule for each other; diluting out LBP and/or the binding molecule; preventing expression of LBP and/or the binding molecule; reducing synthesis of LBP and/or the binding molecule; synthesizing an abnormal LBP and/or binding molecule; synthesizing an alternatively spliced LBP and/or binding molecule; preventing or reducing proper conformational folding of LBP and/or the binding molecule; modulating the binding properties of LBP and/or the binding molecule; interfering with signals that are required to activate or deactivate LBP and/or the binding molecule; activating or deactivating LBP

and/or the binding molecule in such a way as to prevent binding; or interfering with other receptors, ligands or other molecules which are required for the normal synthesis or functioning of LBP and/or the binding molecule. For example, the agent can block the binding site on LDL for LBPs expressed focally in the arterial wall extracellular matrix, or it could block the binding site on an LBP for LDL, or it could be bifunctional, i.e., it could block both binding sites.

Examples of agents include LBP polypeptide, e.g., LBP-1, LBP-2 or LBP-3, or a biologically active fragment or analog thereof; a nucleic acid encoding LBP polypeptide or a biologically active fragment or analog thereof; a nucleic acid encoding an LBP regulatory sequence or a biologically active fragment or analog thereof; a binding molecule for LBP polypeptide; a binding molecule for LBP nucleic acid, the LBP nucleic acid being, e.g., a nucleic acid comprising a regulatory region for LBP or a nucleic acid comprising a structural region for LBP or a biologically active fragment of LBP; an antisense nucleic acid; a mimetic of LBP or a binding molecule; an antibody for LBP or a binding molecule; a metabolite; or an inhibitory carbohydrate or glycoprotein. In certain embodiments, the agent is an antagonist, agonist or super agonist.

Knowledge of the existence of the sequence of the LBPs allows a search for natural or artificial ligands to regulate LDL levels in the treatment of atherosclerosis. In certain embodiments, the agent is a natural ligand for LBP. In certain embodiments, the agent is an artificial ligand for LBP.

By analog is meant a compound that differs from naturally occurring LBP in amino acid sequence or in ways that do not involve sequence, or both. Analogs of the invention generally exhibit at least about 80% homology, preferably at least about 90% homology, more preferably yet at least about 95% homology, and most preferably at least about 98% homology, with substantially the entire sequence of a naturally occurring LBP sequence, preferably with a segment of about 100 amino acid residues, more preferably with a segment of about 50 amino acid residues, more preferably yet with a segment of about 30 amino acid residues, more preferably yet with a segment of about 20 amino acid residues, more preferably yet with a segment of about 10 amino acid residues, more preferably yet with a segment of about 5 amino acid residues, more preferably yet with a segment of about 4 amino acid residues, more preferably yet with a segment of about 3

amino acid residues, and most preferably with a segment of about 2 amino acid residues. Non-sequence modifications include, e.g., in vivo or in vitro chemical derivatizations of LBP. Non-sequence modifications include, e.g., changes in phosphorylation, acetylation, methylation, carboxylation, or glycosylation. Methods for making such modifications are known to those skilled in the art. For example, phosphorylation can be modified by exposing LBP to phosphorylation-altering enzymes, e.g., kinases or phosphatases. Preferred analogs include LBP or biologically active fragments thereof whose sequences differ from the wild-type sequence by one or more conservative amino acid substitutions or by one or more non-conservative amino acid substitutions, deletions, or insertions which do not abolish LBP biological activity. Conservative substitutions typically include the substitution of one amino acid for another with similar characteristics, e.g., substitutions within the following groups: valine, glycine; glycine, alanine; valine, isoleucine, leucine; aspartic acid, glutamic acid; asparagine, glutamine; serine, threonine; lysine, arginine; and phenylalanine, tyrosine. Other examples of conservative substitutions are shown in Table 1.

Table 1

CONSERVATIVE AMINO ACID SUBSTITUTIONS

For Amino Acid	**Code**	**Replace with any of**
Alanine	A	D-Ala, Gly, beta-Ala, L-Cys, D-Cys
Arginine	R	D-Arg, Lys, D-Lys, homo-Arg, D-homo-Arg, Met, Ile, D-Met, D-Ile, Orn, D-Orn, L-NMMA, L-NAME
Asparagine	N	D-Asn, Asp, D-Asp, Glu, D-Glu, Gln, D-Gln
Aspartic Acid	D	D-Asp, D-Asn, Asn, Glu, D-Glu, Gln, D-Gln
Cysteine	C	D-Cys, S-Me-Cys, Met, D-Met, Thr, D-Thr
Glutamine	Q	D-Gln, Asn, D-Asn, Glu, D-Glu, Asp, D-Asp
Glutamic Acid	E	D-Glu, D-Asp, Asp, Asn, D-Asn, Gln, D-Gln
Glycine	G	Ala, D-Ala, Pro, D-Pro, β-Ala Acp
Histidine	H	D-His
Isoleucine	I	D-Ile, Val, D-Val, Leu, D-Leu, Met, D-Met
Leucine	L	D-Leu, Val, D-Val, Leu, D-Leu, Met, D-Met
Lysine	K	D-Lys, Arg, D-Arg, homo-Arg, D-homo-Arg, Met, D-Met, Ile, D-Ile, Orn, D-Orn
Methionine	M	D-Met, S-Me-Cys, Ile, D-Ile, Leu, D-Leu, Val, D-Val

Phenylalanine	F	D-Phe, Tyr, D-Thr, L-Dopa, His, D-His, Trp, D-Trp, Trans-3,4, or 5-phenylproline, cis-3,4, or 5-phenylproline
Proline	P	D-Pro, L-I-thioazolidine-4-carboxylic acid, D- or L-1-oxazolidine-4-carboxylic acid
Serine	S	D-Ser, Thr, D-Thr, allo-Thr, Met, D-Met, Met(O), D-Met(O), L-Cys, D-Cys
Threonine	T	D-Thr, Ser, D-Ser, allo-Thr, Met, D-Met, Met(O), D-Met(O), Val, D-Val
Tryptophan	W	D-Trp, Phe, D-Phe, Tyr, D-Tyr
Tyrosine	Y	D-Tyr, Phe, D-Phe, L-Dopa, His, D-His
Valine	V	D-Val, Leu, D-Leu, Ile, D-Ile, Met, D-Met

Amino acid sequence variants of a protein can be prepared by any of a variety of methods known to those skilled in the art. For example, random mutagenesis of DNA which encodes a protein or a particular domain or region of a protein can be used, e.g., PCR mutagenesis (using, e.g., reduced Taq polymerase fidelity to introduce random mutations into a cloned fragment of DNA; Leung et al., BioTechnique l: ll-15 (1989)), or saturation mutagenesis (by, e.g., chemical treatment or irradiation of single-stranded DNA in vitro, and synthesis of a complementary DNA strand; Mayers et al., Science 229: 242 (1985)). Random mutagenesis can also be accomplished by, e.g., degenerate oligonucleotide generation (using, e.g., an automatic DNA synthesizer to chemically synthesize degenerate sequences; Narang, Tetrahedron 39: 3 (1983); Itakura et al., Recombinant DNA, Proc. 3rd Cleveland Sympos. Macromolecules, ed. A. G. Walton, Amsterdam: Elsevier, pp. 273-289 (1981)). Non-random or directed mutagenesis can be used to provide specific sequences or mutations in specific regions. These techniques can be used to create variants which include, e.g., deletions, insertions, or substitutions, of residues of the known amino acid sequence of a protein. The sites for mutation can be modified individually or in series, e.g., by (i) substituting first with conserved amino acids and then with more radical choices depending upon results achieved, (ii) deleting the target residue, (iii) inserting residues of the same or a different class adjacent to the located site, or (iv) combinations of the above. For example, analogs can be made by in vitro DNA sequence modifications of the sequences of Figs. 2A, 7A, 8A, l0-18 (SEQ ID NOS: l0-18, 45, 46, and 48). For example, in vitro mutagenesis can be used to convert any of these DNA sequences into a sequence which encodes an analog in which one or

more amino acid residues has undergone a replacement, e.g., a conservative replacement as described in Table 1.

Methods for identifying desirable mutations include, e.g., alanine scanning mutagenesis (Cunningham and Wells, Science 244: 1081-1085 (1989)), oligonucleotide-mediated mutagenesis (Adelman et al., DNA, 2: 183 (1983)); cassette mutagenesis (Wells et al., Gene 34: 315 (1985)), combinatorial mutagenesis, and phage display libraries (Ladner et al., PCT International Appln. No. WO88/06630). The LBP analogs can be tested, e.g., for their ability to bind to LDL and/or to an arterial extracellular matrix component, as described herein. Other analogs within the invention include, e.g., those with modifications which increase peptide stability. Such analogs may contain, e.g., one or more non-peptide bonds (which replace the peptide bonds) in the peptide sequence. Also included are, e. g.: analogs that include residues other than naturally occurring L-amino acids, e.g., D-amino acids or nonnaturally occurring or synthetic amino acids, e.g., β or γ amino acids; and cyclic analogs.

Analogs are also meant to include peptides in which structural modifications have been introduced into the peptide backbone so as to make the peptide non-hydrolyzable. Such peptides are particularly useful for oral administration, as they are not digested. Peptide backbone modifications include, e.g., modifications of the amide nitrogen, the α-carbon, the amide carbonyl, or the amide bond, and modifications involving extensions, deletions or backbone crosslinks. For example, the backbone can be modified by substitution of a sulfoxide for the

carbonyl, by reversing the peptide bond, or by substituting a methylene for the carbonyl group. Such modifications can be made by standard procedures known to those skilled in the art. See, e.g., Spatola, A.F., "Peptide Backbone Modifications: A Structure-Activity Analysis of Peptides Containing Amide Bond Surrogates, Conformational Constraints, and Related Backbone Replacements," in Chemistry and Biochemistry of Amino Acids, Peptides and Proteins, Vol. 7, pp. 267-357, B. Weinstein (ed.), Marcel Dekker, Inc., New York (1983).

An analog is also meant to include polypeptides in which one or more of the amino acid residues include a substituent group, or polypeptides which are fused with another compound, e.g., a compound to increase the half-life of the polypeptide, e.g.,

polyethylene glycol.

By fragment is meant some portion of the naturally occurring LBP polypeptide. Preferably, the fragment is at least about 100 amino acid residues, more preferably at least about 50 amino acid residues, more preferably yet at least about 30 amino acid residues, more preferably yet at least about 20 amino acid residues, more preferably yet at least about 5 amino acid residues, more preferably yet at least about 4 amino acid residues, more preferably yet at least about 3 amino acid residues, and most preferably at least about 2 amino acid residues in length. Fragments include, e.g., truncated secreted forms, proteolytic fragments, splicing fragments, other fragments, and chimeric constructs between at least a portion of the relevant gene, e.g., LBP-1, LBP-2 or LBP-3, and another molecule. Fragments of LBP can be generated by methods known to those skilled in the art. In certain embodiments, the fragment is biologically active. The ability of a candidate fragment to exhibit a biological activity of LBP can be assessed by methods known to those skilled in the art. For example, LBP fragments can be tested for their ability to bind to LDL and/or to an arterial extracellular matrix structural component, as described herein. Also included are LBP fragments containing residues that are not required for biological activity of the fragment or that result from alternative mRNA splicing or alternative protein processing events.

Fragments of a protein can be produced by any of a variety of methods known to those skilled in the art, e.g., recombinantly, by proteolytic digestion, or by chemical synthesis. Internal or terminal fragments of a polypeptide can be generated by removing one or more nucleotides from one end (for a terminal fragment) or both ends (for an internal fragment) of a nucleic acid which encodes the polypeptide. Expression of the mutagenized DNA produces polypeptide fragments. Digestion with "end-nibbling" endonucleases can thus generate DNAs which encode an array of fragments. DNAs which encode fragments of a protein can also be generated; e.g., by random shearing, restriction digestion or a combination of the above-discussed methods. For example, fragments of LBP can be made by expressing LBP DNA which has been manipulated in vitro to encode the desired fragment, e.g., by restriction digestion of any of the DNA sequences of Figs. 2A, 7A, 8A, 10-18 (SEQ ID NOS: 10-18, 45, 46, and 48).

Fragments can also be chemically synthesized using techniques known in the art,

e.g., conventional Merrifield solid phase f-Moc or t-Boc chemistry for example, peptides of the present invention can be arbitrarily divided into fragments of desired length with no overlap of the fragments, or divided into overlapping fragments of a desired length.

An LBP or a biologically active fragment or analog thereof, or a binding molecule or a biologically active fragment or analog thereof, can, e.g., compete with its cognate molecule for the binding site on the complementary molecule, and thereby reduce or eliminate binding between LBP and the cellular binding molecule. LBP or a binding molecule can be obtained, e.g., from purification or secretion of naturally occurring LBP or binding molecule, from recombinant LBP or binding molecule, or from synthesized LBP or binding molecule.

Therefore, methods for generating analogs and fragments and testing them for activity are known to those skilled in the art.

An agent can also be a nucleic acid used as an antisense molecule. Antisense therapy is meant to include, e.g., administration or in situ generation of oligonucleotides or their derivatives which specifically hybridize, e.g., bind, under cellular conditions, with the cellular mRNA and/or genomic DNA encoding an LBP polypeptide, or mutant thereof, so as to inhibit expression of the encoded protein, e.g., by inhibiting transcription and/or translation. The binding may be by conventional base pair complementarity, or, for example, in the case of binding to DNA duplexes, through specific interactions in the major groove of the double helix.

In certain embodiments, the antisense construct binds to a naturally-occurring sequence of an LBP gene which, e.g., is involved in expression of the gene. These sequences include, e.g., promoter, start codons, stop codons, and RNA polymerase binding sites. In other embodiments, the antisense construct binds to a nucleotide sequence which is not present in the wild type gene. For example, the antisense construct can bind to a region of an LBP gene which contains an insertion of an exogenous, non-wild type sequence. Alternatively, the antisense construct can bind to a region of an LBP gene which has undergone a deletion, thereby bringing two regions of the gene together which are not normally positioned together and which, together, create a non-wild type sequence. When administered in vivo to a subject, antisense constructs which bind to non-wild type sequences provide the advantage of inhibiting the expression of a mutant

LBP gene, without inhibiting expression of any wild type LBP gene.

An antisense construct of the present invention can be delivered, e.g., as an expression plasmid which, when transcribed in the cell, produces RNA which is complementary to at least a unique portion of the cellular mRNA which encodes an LBP polypeptide. An alternative is that the antisense construct is an oligonucleotide which is generated ex vivo and which, when introduced into the cell causes inhibition of expression by hybridizing with the mRNA (duplexing) and/or genomic sequences (triplexing) of an LBP gene. Such oligonucleotides are preferably modified oligonucleotides which are resistant to endogenous nucleases, e. g. exonucleases and/or endonucleases, and are therefore stable in vivo. Exemplary nucleic acid molecules for use as antisense oligonucleotides are phosphoramidate, phosphothioate, phosphorodithioates and methylphosphonate analogs of DNA and peptide nucleic acids (PNA). (See also U.S. Patents 5,176,996; 5,264,564; and 5,256,775). Additionally, general approaches to constructing oligomers useful in antisense therapy have been reviewed. (See, e.g., Van der Krol et al., Biotechniques 6: 958-976, (1988); Stein et al., Cancer Res. 48: 2659-2668 (1988)).

By mimetic is meant a molecule which resembles in shape and/or charge distribution LBP or a binding molecule. The mimetic can be a peptide or a non-peptide. Mimetics can act as therapeutic agents because they can, e.g., competitively inhibit binding of LBP to a binding molecule. By employing, e.g., scanning mutagenesis, e.g., alanine scanning mutagenesis, linker scanning mutagenesis or saturation mutagenesis, to map the amino acid residues of a particular LBP polypeptide involved in binding a binding molecule, peptide mimetics, e.g., diazepine or isoquinoline derivatives, can be generated which mimic those residues in binding to a binding molecule, and which therefore can inhibit binding of the LBP to a binding molecule and thereby interfere with the function of LBP. Non-hydrolyzable peptide analogs of such residues can be generated using, e.g., benzodiazepine (see, e.g., Freidinger et al., in Peptides: Chemistry and Biology, G. R. Marshall ed., ESCOM Publisher: Leiden, Netherlands (1988)); azepine (see, e.g., Huffman et al., in Peptides: Chemistry and Biology, G. R. Marshall ed., ESCOM Publisher: Leiden, Netherlands (1988)); substituted gamma lactam rings (see, e.g., Garvey et al., in Peptides: Chemistry and Biology, G. R. Marshall ed., ESCOM

Publisher: Leiden, Netherlands (1988)); keto-methylene pseudopeptides (see, e.g., Ewenson et al., J. Med. Chem. 29: 295 (1986); Ewenson et al., in Peptides: Structure and Function (Proceedings of the 9th American Peptide Symposium) Pierce Chemical Co. Rockland, IL (1985)); β-turn dipeptide cores (see, e.g., Nagai et al., Tetrahedron Lett. 26: 647 (1985); Sato et al., J. Chem. Soc. Perkin Trans. 1: 1231 (1986)); or β-aminoalcohols (see, e.g., Gordon et al., Biochem. Biophys. Res. Commun. 126:419 (1985); Dann et al., Biochem. Biophys. Res. Commun. 134:71 (1986)).

Antibodies are meant to include antibodies against any moiety that directly or indirectly affects LBP metabolism. The antibodies can be directed against, e.g., LBP or a binding molecule, or a subunit or fragment thereof. For example, antibodies include anti-LBP-1, LBP-2 or LBP-3 antibodies; and anti-binding molecule antibodies. Antibody fragments are meant to include, e.g., Fab fragments, Fab' fragments, $F(ab')_2$ fragments, F(v) fragments, heavy chain monomers, heavy chain dimers, heavy chain trimers, light chain monomers, light chain dimers, light chain trimers, dimers consisting of one heavy and one light chain, and peptides that mimic the activity of the anti-LBP or anti-binding molecule antibodies. For example, Fab_2' fragments of the inhibitory antibody can be generated through, e.g., enzymatic cleavage. Both polyclonal and monoclonal antibodies can be used in this invention. Preferably, monoclonal antibodies are used. Natural antibodies, recombinant antibodies or chimeric-antibodies, e.g., humanized antibodies, are included in this invention. Preferably, humanized antibodies are used when the subject is a human. Most preferably, the antibodies have a constant region derived from a human antibody and a variable region derived from an inhibitory mouse monoclonal antibody. Production of polyclonal antibodies to LBP is described in Example 6. Monoclonal and humanized antibodies are generated by standard methods known to those skilled in the art. Monoclonal antibodies can be produced, e.g., by any technique which provides antibodies produced by continuous cell lines cultures. Examples include the hybridoma technique (Kohler and Milstein, Nature 256: 495-497 (1975), the trioma technique, the human B-cell hybridoma technique (Kozbor et al., Immunology Today 4:72 (1983)), and the EBV-hybridoma technique to produce human monoclonal antibodies (Cole et al., in Monoclonal Antibodies and Cancer Therapy, A. R. Lisa, Inc., pp. 77-96 (1985)). Preferably, humanized antibodies are raised through conventional

production and harvesting techniques (Berkower, I., Curr. Opin. Biotechnol. 7:622-628 (1996); Ramharayan and Skaletsky, Am. Biotechnol. Lab 13:26-28 (1995)). In certain preferred embodiments, the antibodies are raised against the LBP, preferably the LDL-binding site, and the Fab fragments produced. These antibodies, or fragments derived therefrom, can be used, e.g., to block the LDL-binding sites on the LBP molecules.

Agents also include inhibitors of a molecule that are required for synthesis, post-translational modification, or functioning of LBP. and/or a binding molecule, or activators of a molecule that inhibits the synthesis or functioning of LBP and/or the binding molecule. Agents include, e.g., cytokines, chemokines, growth factors, hormones, signaling components, kinases, phosphatases, homeobox proteins, transcription factors, editing factors, translation factors and post-translation factors or enzymes. Agents are also meant to include ionizing radiation, non-ionizing radiation, ultrasound and toxic agents which can, e.g., at least partially inactivate or destroy LBP and/or the binding molecule.

An agent is also meant to include an agent which is not entirely LBP specific. For example, an agent may alter other genes or proteins related to arterial plaque formation. Such overlapping specificity may provide additional therapeutic advantage.

The invention also includes the agent so identified as being useful in treating atherosclerosis.

The invention also includes a method for evaluating an agent for the ability to alter the binding of LBP polypeptide to a binding molecule. An agent is provided. An LBP polypeptide is provided. A binding molecule is provided. The agent, LBP polypeptide and binding molecule are combined. The formation of a complex comprising the LBP polypeptide and binding molecule is detected. An alteration in the formation of the complex in the presence of the agent as compared to in the absence of the agent is indicative of the agent altering the binding of the LBP polypeptide to the binding molecule.

In preferred embodiments, the LBP polypeptide is LBP-1, LBP-2 or LBP-3. Examples of a binding molecule include native LDL, modified LDL, e.g., methylated LDL or oxidized LDL, and arterial extracellular matrix structural components.

Altering the binding includes, e.g., inhibiting or promoting the binding. The

efficacy of the agent can be assessed, e.g., by generating dose response curves from data obtained using various concentrations of the agent. Methods for determining formation of a complex are standard and are known to those skilled in the art, e.g., affinity coelectrophoresis (ACE) assays or ELISA assays as described herein.

The invention also includes the agent so identified as being able to alter the binding of an LBP polypeptide to a binding molecule.

The invention also includes a method for evaluating an agent for the ability to bind to an LBP polypeptide. An agent is provided. An LBP polypeptide is provided. The agent is contacted with the LBP polypeptide. The ability of the agent to bind to the LBP polypeptide is evaluated. Preferably, the LBP polypeptide is LBP-1, LBP-2 or LBP-3. Binding can be determined, e.g., by measuring formation of a complex by standard methods known to those skilled in the art, e.g., affinity coelectrophoresis (ACE) assays or ELISA assays as described herein.

The invention also includes the agent so identified as being able to bind to LBP polypeptide.

The invention also includes a method for evaluating an agent for the ability to bind to a nucleic acid encoding an LBP regulatory sequence. An agent is provided. A nucleic acid encoding an LBP regulatory sequence is provided. The agent is contacted with the nucleic acid. The ability of the agent to bind to the nucleic acid is evaluated. Preferably, the LBP regulatory sequence is an LBP-1, LBP-2 or LBP-3 regulatory sequence. Binding can be determined, e.g., by measuring formation of a complex by standard methods known to those skilled in the art, e.g., DNA mobility shift assays, DNase I footprint analysis Molecular Biology, The invention being able to bind sequence. (Ausubel et al., ed., Current Protocols in John Wiley & Sons, New York, NY, (1989)).

The invention also includes the agent so identified as to a nucleic acid encoding an LBP regulatory sequence.

The invention also includes a method for treating atherosclerosis in an animal. An animal in need of treatment for atherosclerosis is provided. An agent capable of altering an aspect of LBP structure or metabolism is provided. The agent is administered to the animal in a therapeutically effective amount such that treatment of the atherosclerosis

occurs.

In certain preferred embodiments, the agent is an LBP polypeptide, e.g., LBP-1, LBP-2 or LBP-3, or a biologically active fragment or analog thereof. The agent can be, e.g., the polypeptide as set forth in SEQ ID NOS: 1-9, 43, 44, and 47. Preferably, the agent is a polypeptide of no more than about 100 amino acid residues in length, more preferably of no more than about 50 amino acid residues, more preferably yet of no more than about 30 amino acid residues, more preferably yet of no more than about 20 amino acid residues, more preferably yet of no more than about 10 amino acid residues, more preferably yet of no more than about 5 amino acid residues, more preferably yet of no more than about 4 amino acid residues, more preferably yet of no more than about 3 amino acid residues, and most preferably of no more than about 2 amino acid residues. Preferably, the polypeptide includes at least about 20% acidic amino acid residues, more preferably yet at least about 40% acidic amino acid residues, more preferably yet at least about 60% acidic amino acid residues, more preferably yet at least about 80% acidic amino acid residues, more preferably yet at least about 90% acidic amino acid residues, more preferably yet at least about 95% acidic amino acid residues, and most preferably at least about 98% acidic amino acid residues. Acidic amino acid residues include aspartic acid and glutamic acid. An example of such an LBP poly-peptide is BHF-1, which is a 20 amino acid length fragment of human or rabbit LBP-1 which contains amino acid residues 14 through 33. See Fig. 9 (SEQ ID NO: 9). 45% of the amino acid residues of BHF-1 are acidic. The invention also includes biologically active fragments and analogs of BHF-1.

Other preferred acidic regions from the LBPs are amino acid residues 329 through 343 (SEQ ID NO: 19), 329 through 354 (SEQ ID NO: 20), 344 through 354 (SEQ ID NO: 21), and 529 through 538 (SEQ ID NO: 22) of human LBP-2 as depicted in Fig. 7A (SEQ. ID NO: 43); amino acid residues 14 through 43 (SEQ ID NO: 23)and 38 through 43 (SEQ ID NO: 24) of rabbit or human LBP-1 as depicted in Fig. 1 (SEQ ID NO: 1) and Fig. 6 (SEQ ID NO: 6); amino acid residues 338 through 353 (SEQ ID NO: 25), 338 through 365 (SEQ ID NO: 26), 354 through 365 (SEQ ID NO: 27), and 444 through 453 (SEQ ID NO: 28) of rabbit LBP-2 as depicted in Fig. 2A (SEQ ID NO: 47); amino acid residues 96 through 110 (SEQ ID NO: 29) of rabbit LBP-3 as depicted in Fig. 5 (SEQ ID

NO: 5); and amino acid residues 69-75 (SEQ ID NO: 41) of human LBP-3 as depicted in Fig. 8A (SEQ ID NO: 44). The invention is also meant to include biologically active fragments and analogs of any of these polypeptides.

Other examples of agents include homopolymers and heteropolymers of any amino acid or amino acid analog. In certain preferred embodiments, the agent is a homopolymer of an acidic amino acid or analog thereof. In certain embodiments, the agent is a heteropolymer of one or more acidic amino acids and one or more other amino acids, or analogs thereof. For example, agents include poly(glu), poly(asp), poly(glu asp), poly(glu N), poly (asp N) and poly(glu asp N). By N is meant any amino acid, or analog thereof, other than glu or asp. By poly(glu asp) is meant all permutations of glu and asp for a given length peptide. A preferred peptide is poly(glu) of no more than about 10 amino acids in length, preferably about 7 amino acids in length.

In certain preferred embodiments, the agent is an LBP nucleic acid or a biologically active fragment or analog thereof, e.g., a nucleic acid encoding LBP-1, LBP-2 or LBP-3 polypeptide, or a biologically active fragment or analog thereof. The agent can be, e.g., a nucleic acid comprising a nucleotide sequence as set forth in SEQ ID NOS: 10-18, 45, 46, and 48. In other embodiments, the agent is an antisense molecule, e.g., one which can bind to an LBP gene sequence.

Treating is meant to include, e.g., preventing, treating, reducing the symptoms of, or curing the atherosclerosis. Administration of the agent can be accomplished by any method which allows the agent to reach the target area, e.g., a target cell or the extracellular matrix. These methods include, e.g., injection, deposition, implantation, suppositories, oral ingestion, inhalation, topical administration, or any other method of administration where access to the target area by the agent is obtained. Injections can be, e.g., intravenous, intradermal, subcutaneous, intramuscular or intraperitoneal. Implantation includes inserting implantable drug delivery systems, e.g., microspheres, hydrogels, polymeric reservoirs, cholesterol matrices, polymeric systems, e.g., matrix erosion and/or diffusion systems and non-polymeric systems, e.g., compressed, fused or partially fused pellets. Suppositories include glycerin suppositories. Oral ingestion doses can be enterically coated. Inhalation includes administering the agent with an aerosol in an inhalator, either alone or attached to a carrier that can be absorbed.

Administration of the agent can be alone or in combination with other therapeutic agents. In certain embodiments, the agent can be combined with a suitable carrier, incorporated into a liposome, or incorporated into a polymer release system.

In certain embodiments of the invention, the administration can be designed so as to result in sequential exposures to the agent over some time period, e.g., hours, days, weeks, months or years. This can be accomplished by repeated administrations of the agent by one of the methods described above, or alternatively, by a controlled release delivery system in which the agent is delivered to the animal over a prolonged period without repeated administrations. By a controlled release delivery system is meant that total release of the agent does not occur immediately upon administration, but rather is delayed for some time. Release can occur in bursts or it can occur gradually and continuously. Administration of such a system can be, e.g., by long acting oral dosage forms, bolus injections, transdermal patches or subcutaneous implants.

Examples of systems in which release occurs in bursts include, e.g., systems in which the agent is entrapped in liposomes which are encapsulated in a polymer matrix, the liposomes being sensitive to a specific stimulus, e.g., temperature, pH, light, magnetic field, or a degrading enzyme, and systems in which the agent is encapsulated by an ionically-coated microcapsule with a microcapsule core-degrading enzyme. Examples of systems in which release of the agent is gradual and continuous include, e.g., erosional systems in which the agent is contained in a form within a matrix, and diffusional systems in which the agent permeates at a controlled rate, e.g., through a polymer. Such sustained release systems can be, e.g., in the form of pellets or capsules.

The agent can be suspended in a liquid, e.g., in dissolved form or colloidal form. The liquid can be a solvent, partial solvent or non-solvent. In many cases water or an organic liquid can be used.

The agent can be administered prior to or subsequent to the appearance of atherosclerosis symptoms. In certain embodiments, the agent is administered to patients with familial histories of atherosclerosis, or who have phenotypes that may indicate a predisposition to atherosclerosis, or who have been diagnosed as having a genotype which predisposes the patient to atherosclerosis, or who have other risk factors, e.g., hypercholesterolemia, hypertension or smoking.

The agent is administered to the animal in a therapeutically effective amount. By therapeutically effective amount is meant that amount which is capable of at least partially preventing or reversing atherosclerosis. A therapeutically effective amount can be determined on an individual basis and will be based, at least in part, on consideration of the species of animal, the animal's size, the animal's age, the agent used, the type of delivery system used, the time of administration relative to the onset of atherosclerosis symptoms, and whether a single, multiple, or controlled release dose regimen is employed. A therapeutically effective amount can be determined by one of ordinary skill in the art employing such factors and using no more than routine experimentation.

Preferably, the concentration of the agent is at a dose of about 0.1 to about 1000 mg/kg body weight/day, more preferably at about 0.1 to about 500 mg/kg/day, more preferably yet at about 0.1 to about 100 mg/kg/day, and most preferably at about 0.1 to about 5 mg/kg/day. The specific concentration partially depends upon the particular agent used, as some are more effective than others. The dosage concentration of the agent that is actually administered is dependent at least in part upon the final concentration that is desired at the site of action, the method of administration, the efficacy of the particular agent, the longevity of the particular agent, and the timing of administration relative to the onset of the atherosclerosis symptoms. Preferably, the dosage form is such that it does not substantially deleteriously affect the animal. The dosage can be determined by one of ordinary skill in the art employing such factors and using no more than routine experimentation.

In certain embodiments, various gene constructs can be used as part of a gene therapy protocol to deliver nucleic acids encoding an agent, e.g., either an agonistic or antagonistic form of an LBP polypeptide. For example, expression vectors can be used for in vivo transfection and expression of an LBP polypeptide in particular cell types so as to reconstitute the function of, or alternatively, abrogate the function of, LBP polypeptide in a cell in which non-wild type LBP is expressed. Expression constructs of the LBP polypeptide, and mutants thereof, may be administered in any biologically effective carrier, e.g., any formulation or composition capable of effectively delivering the LBP gene to cells in vivo. Approaches include, e.g., insertion of the subject gene in viral vectors including, e.g., recombinant retroviruses, adenovirus, adeno-associated

virus, and herpes simplex virus-1, or recombinant bacterial or eukaryotic plasmids. Viral vectors infect or transduce cells directly; plasmid DNA can be delivered with the help of, for example, cationic liposomes (lipofectin™ (Life Technologies, Inc., Gaithersburg, MD) or derivatized (e.g. antibody conjugated), polylysine conjugates, gramacidin S, artificial viral envelopes or other such intracellular carriers, as well as direct injection of the gene construct or $Ca_3,(P0_4)_2$ precipitation carried out in vivo. The above-described methods are known to those skilled in the art and can be performed without undue experimentation. Since transduction of appropriate target cells represents the critical first step in gene therapy, choice of the particular gene delivery system will depend on such factors as the phenotype of the intended target and the route of administration, e.g., locally or systemically. Administration can be directed to one or more cell types, and to one or more cells within a cell type, so as to be therapeutically effective, by methods that are known to those skilled in the art. In a preferred embodiment, the agent is administered to arterial wall cells of the animal. For example, a genetically engineered LBP gene is administered to arterial wall cells. In certain embodiments, administration is done in a prenatal animal or embryonic cell. It will be recognized that the particular gene construct provided for in vivo transduction of LBP expression is also useful for in vitro transduction of cells, such as for use in the diagnostic assays described herein.

In certain embodiments, therapy of atherosclerosis is performed with antisense nucleotide analogs of the genes which code for the LBPs. Preferably, the antisense nucleotides have non-hydrolyzable "backbones," e.g., phosphorothioates, phosphorodithioates or methylphosphonates. The nucleoside base sequence is complementary to the sequence of a portion of the gene coding for, e.g., LBP-1, 2 or 3. Such a sequence might be, e.g., ATTGGC if the gene sequence for the LBP is TAACCG. One embodiment of such therapy would be incorporation of an antisense analog of a portion of one of the LBP genes in a slowrelease medium, e.g., polyvinyl alcohol, which is administered, e.g., by subcutaneous injection, so as to release the antisense nucleotide analog over a period of weeks or months. In another embodiment, the antisense analog is incorporated into a polymeric matrix, e.g., polyvinyl alcohol, such that the gel can be applied locally to an injured arterial wall to inhibit LBP synthesis and prevent LDL accumulation, e.g., after angioplasty or atherectomy.

The invention also includes a method for treating an animal at risk for atherosclerosis. An animal at risk for atherosclerosis is provided. An agent capable of altering an aspect of LBP structure or metabolism is provided. The agent is administered to the animal in a therapeutically effective amount such that treatment of the animal occurs. Being at risk for atherosclerosis can result from, e.g., a family history of atherosclerosis, or phenotypic symptoms which predispose to atherosclerosis, e.g., having hypercholesterolemia, hypertension or smoking.

The invention also includes a method for treating a cell having an abnormality in structure or metabolism of LBP. A cell having an abnormality in structure or metabolism of LBP is provided. An agent capable of altering an aspect of LBP structure or metabolism is provided. The agent is administered to the cell in a therapeutically effective amount such that treatment of the cell occurs.

In certain embodiments, the cell is obtained from a cell culture or tissue culture or an embryo fibroblast. The cell can be, e.g., part of an animal, e.g., a natural animal or a nonhuman transgenic animal. Preferably, the LBP is LBP-1, LBP-2 or LBP-3.

The invention also includes a pharmaceutical composition for treating atherosclerosis in an animal comprising a therapeutically effective amount of an agent, the agent being capable of altering an aspect of LBP metabolism or structure in the animal so as to result in treatment of the atherosclerosis, and a pharmaceutically acceptable carrier. Pharmaceutically acceptable carriers include, e.g., saline, liposomes and lipid emulsions.

In certain preferred embodiments, the agent of the pharmaceutical composition is an LBP polypeptide, e.g., LBP-1, LBP-2 or LBP-3, or a biologically active fragment or analog thereof. The agent can be, e.g., the polypeptide as set forth in SEQ ID NOS: 1-9, 43, 44, and 47. Preferably, the agent is a polypeptide of no more than about 100 amino acid residues in length, more preferably of no more than about 50 amino acid residues, more preferably yet of no more than about 30 amino acid residues, more preferably yet of no more than about 20 amino acid residues, more preferably yet of no more than about 10 amino acid residues, more preferably yet of no more than about 5 amino acid residues, more preferably yet of no more than about 4 amino acid residues, more preferably yet of no more than about 3 amino acid residues, and most preferably of no more than about 2

amino acid residues. Preferably, the polypeptide includes at least about 20% acidic amino acid residues, more preferably yet at least about 40% acidic amino acid residues, more preferably yet at least about 60% acidic amino acid residues, more preferably yet at least about 80% acidic amino acid residues, more preferably yet at least about 90% acidic amino acid residues, more preferably yet at least about 95% acidic amino acid residues, and most preferably at least about 98%acidic amino acid residues.

In certain preferred embodiments, the agent is an LBP nucleic acid, e.g., a nucleic acid encoding LBP-1, LBP-2 or LBP-3 polypeptide, or a biologically active fragment or analog thereof. The agent can be, e.g., a nucleic acid comprising a nucleotide sequence as set forth in SEQ ID NOS: 10-18, 45, 46, and 48.

The invention also includes a vaccine composition for treating atherosclerosis in an animal comprising a therapeutically effective amount of an agent, the agent being capable of altering an aspect of LBP metabolism or structure in the animal so as to result in treatment of the atherosclerosis, and a pharmaceutically acceptable carrier.

The invention also includes a method for diagnosing atherosclerotic lesions in an animal. An animal is provided. A labeled agent capable of binding to LBP present in atherosclerotic lesions is provided. The labeled agent is administered to the animal under conditions which allow the labeled agent to interact with the LBP so as to result in labeled LBP. The localization or quantification of the labeled LBP is determined by imaging so as to diagnose the presence of atherosclerotic lesions in the animal.

Preferably, the LBP is LBP-1, LBP-2 or LBP-3. The imaging can be performed by standard methods known to those skilled in the art, including, e.g., magnetic resonance imaging, gamma camera imaging, single photon emission computed tomographic (SPECT) imaging, or positron emission tomography (PET).

Preferably, agents that bind tightly to LBPs in atherosclerotic lesions are used for atherosclerotic imaging and diagnosis. The agent is radiolabeled with, e.g., ^{99m}Tc or another isotope suitable for clinical imaging by gamma camera, SPECT, PET scanning or other similar technology. Since LBPs occur in very early lesions, such imaging is more sensitive than angiography or ultrasound for locating very early lesions which do not yet impinge on the arterial lumen to cause a visible bulge or disturbed flow. In addition to locating both early and more developed lesions, the imaging agents which bind to LBPs

can also be used to follow the progress of atherosclerosis, as a means of evaluating the effectiveness of both dietary and pharmacological treatments.

Thus, a diagnostic embodiment of the invention is the adaptation of, e.g., a peptide complementary to one of the LBPs, by radiolabeling it and using it as an injectable imaging agent for detection of occult atherosclerosis. The peptide is selected from those known to bind to LBPs, e.g., RRRRRRR or KKLKLXX, or any other polycationic peptide which binds to the highly electronegative domains of the LBPs. For extracorporeal detection with a gamma scintillation (Anger) camera, technetium-binding ligands, e.g., CGC, GGCGC, or GGCGCF, can be incorporated into the peptides at the N-terminus or C-terminus for ^{99m}Tc labeling. For external imaging by magnetic resonance imaging (MRI), e.g., the gadolinium-binding chelator, diethylene triamine penta-acetic acid (DTPA), is covalently bound to the N- or C-terminus of the peptides. In yet other embodiments, the LBP-binding peptides are covalently bound, e.g., to magnetic ion oxide particles by standard methods known to those skilled in the art, e.g., conjugating the peptides with activated polystyrene resin beads containing magnetic ion oxide.

The invention also includes a method for immunizing an animal against an LBP, e.g., LBP-1, LBP-2 or LBP-3, or fragment or analog thereof. An animal having LDL is provided. An LBP or fragment or analog thereof is provided. The LBP or fragment or analog thereof is administered to the animal so as to stimulate antibody production by the animal to the LBP or fragment or analog thereof such that binding of the LBP to the LDL is altered, e.g., decreased or increased.

The invention also includes a method of making a fragment or analog of LBP polypeptide, the fragment or analog having the ability to bind to modified LDL and native LDL. An LBP polypeptide is provided. The sequence of the LBP polypeptide is altered. The altered LBP polypeptide is tested for the ability to bind to modified LDL, e.g., methylated LDL, oxidized LDL, acetylated LDL, cyclohexanedione-treated LDL (CHD-LDL), and to native LDL.

The fragments or analogs can be generated and tested for their ability to bind to these modified LDLs and to native LDL, by methods known to those skilled in the art, e.g., as described herein. Preferably, they are tested for their ability to bind to methylated

LDL and native LDL. The binding activity of the fragment or analog can be greater or less than the binding activity of the native LBP. Preferably, it is greater. In preferred embodiments, the LBP is LBP-1, LBP-2 or LBP-3.

The invention also includes a method for isolating a cDNA encoding an LBP. A cDNA library is provided. The cDNA library is screened for a cDNA encoding a polypeptide which binds to native LDL and modified LDL, e.g., methylated LDL or oxidized LDL. The cDNA which encodes this polypeptide is isolated, the cDNA encoding an LBP.

Atherosclerosis in a hyperlipidemic subject can be reduced following the generation of an immune response in the subject by immunization with LBPs. Numerous immunotherapeutic products can be used to generate antibodies that will block the binding between LDL and LBPs.

The injection of one or more LBPs can result in the production of anti-LBP antibodies, resulting in a reduction in ,e.g., aortic atherosclerosis. This effect is thought to be mediated by an inhibition of LBP binding to LDL. LBP immunogens that can be used in the invention include human LBPs, non-human LBPs, recombinant LBPs, and proteins structurally related to the LBPs described herein, e.g. non-naturally occurring proteins that differ from a naturally occurring LBP at one or more amino acid residues. In addition to full length proteins, injecting one or more peptides that include an LBP domain can generate an effective immune response. For example, the injection of a peptide comprising an LBP domain having LDL-binding activity can cause an organism to make antibodies to the LBP binding sites for LDL. These peptide immunogens can include sequences derived from human LBPs, non-human LBPs, recombinant LBPs, and proteins structurally related to the LBPs described herein.

Modifications can be made to a protein or peptide immunogen of the invention to increase its immunogenicity. The immunogen can be conjugated or coupled with a carrier, e.g. a Cholera toxin B chain or monoclonal antibodies. The immunogen can be precipitated with aluminum salts or cross-linked with formaldehyde or other aldehydes. The protein may be mixed with a physiologically acceptable diluent such as water, phosphate buffered saline, or saline. The composition may further include an adjuvant. In addition to RIBI adjuvant, adjuvants such as incomplete Freund's adjuvant, aluminum

phosphate, aluminum hydroxide are all well known in the art. Adjustments in the adjuvant of the invention can be made to affect the immunogenicity of the peptide or protein. Examples of such modifications include using: aluminum salts; cytokines; MF59 (microfluidized emulsion of oil and surfactants); SAF-1 (oil-based emulsion); saponin derivatives; polymers (such as polyphosphazene); and bacterial toxins. Additional descriptions of antigenic protein-adjuvant combinations are described in WO 99/54452 (herein incorporated by reference) and WO 99/49890 (herein incorporated by reference).

In addition to delivery of the proteins and peptides described above, numerous other delivery systems can be used to generate the anti-atherosclerotic immunity of the invention. The LBP immunogen can be delivered either directly as a protein antigen or alternatively as a nucleic acid that encodes the protein antigen. The immunotherapeutic products of the invention, either protein or nucleic acid, can be delivered by numerous delivery routes. These include injection, deposition, implantation, suppositories, oral ingestion, inhalation (e.g., delivery via a nasal spray), and topical administration (e.g., delivery via a skin patch).

A nucleic acid encoding an immunogen of the invention can be directly administered, for example by injection, to tissues and expressed as a protein. The DNA or RNA can be either associated with a delivery vehicle (e.g., viruses, bacteria, liposomes, and gold beads) or naked (free from association with transfection-facilitating proteins, viral particles, liposomal formulations, charged lipids and calcium phosphate precipitating). The nucleic acid can optionally include a promoter, e.g. a viral promoter. The immunogen encoded by the nucleic acid is produced in the host, resulting in the generation of an immune response. Methods for the delivery of nucleic acid sequences encoding therapeutic proteins and peptides are described in detail by Felgner et al. (U.S. 5,580,859; herein incorporated by reference) and Barbet et al. (U.S. 6,025, 338: ; herein incorporated by reference). Vaccine compositions of viral liposomes comprising a nucleic acid, e.g. an RNA, encoding a protein antigen are described in WO 99/52503 (herein incorporated by reference). Proteins and nucleic acids encoding peptides can also be delivered to an individual by their encapsulation in liposomes, microparticles, and ISCOMS, all of which are well known in the art (see, e.g., U.S. 6,013,258, herein incorporated by reference).

A nucleic acid encoding an immunogen of the invention can also be included in the genome of a plant, so as to result in the production of the immunogen by plant tissues. The genetically modified plant may then consumed by an individual, resulting in the ingestion of the immunogen and the generation of an anti-LBP immune response. Methodology for the generation and usage of edible plant vaccines is described in WO 99/54452 (herein incorporated by reference).

Numerous plants may be useful for the production of an edible vaccine, including: tobacco, tomato, potato, eggplant, pepino, yam, soybean, pea, sugar beet, lettuce, bell pepper, celery, carrot, asparagus, onion, grapevine, muskmelon, strawberry, rice, sunflower, rapeseed/canola, wheat, oats, maize, cotton, walnut, spruce/conifer, poplar and apple. The edible vaccine can include a plant cell transformed with a nucleic acid construct comprising a promoter and a sequence encoding an LBP. The sequence may optionally encode a chimeric protein, comprising a cholera toxin subunit B peptide fused to the LBP peptide. Preferred plant promoters of the invention include CaMV 35S, patatin, mas, and granule-bound starch synthase promoters. Additional useful promoters and enhancers are described in WO 99/54452.

The edible vaccine of the invention can be administered to a mammal suffering from or at risk of atherosclerosis. Preferably, an edible vaccine is administered orally, e.g. consuming a transgenic plant of the invention. The transgenic plant can be in the form of a plant part, extract, juice, liquid, powder, or tablet. The edible vaccine can also be administered via an intranasal route.

Microorganisms, e.g., attenuated viruses or bacteria, can be used in the invention by including a nucleic acid encoding an LBP immunogen in the genome of the microorganism. This modified vector can then be delivered to a host, resulting in the *in vivo* production of the immunogen. The immune response generated by these vectors is expected to result in anti-atherosclerotic immunity. Nucleic acid molecules are inserted into microorganism genomes by standard methods known in the art (U.S. 5,866,136 and U.S. 6,025,164, both of which are herein incorporated by reference)

The anti-atherosclerotic methods of the invention are directed to treating a subject, e.g., a human, primate, horse, dog, cat, or goat, at risk for atherosclerosis by stimulating an anti-LBP response in the subject by immunotherapy. The LBP proteins

and peptides of the invention may be delivered to the subject by the numerous delivery systems described herein. The immunotherapy may comprise an initial immunization followed by additional, e.g. one, two, or three, boosters.

The invention also includes a method of treating a subject at risk for atherosclerosis by (1) providing a subject at risk for atherosclerosis and (2) administering to the subject one or more of the following: (a) an LBP protein or fragment or analog thereof and an adjuvant; (b) a nucleic acid encoding an LBP protein; (c) a virus or bacteria comprising a nucleic acid encoding an LBP protein; and (d) an edible plant comprising a nucleic acid encoding an LBP protein. The LBP protein used in this method can be any LBP described herein, e.g., LBP-1, LBP-2, or LBP-3. A combination of more than one nucleic acid or LBP protein or fragment or analog thereof can be administered to the subject. For example, combinations of LBP proteins, or nucleic acids encoding LBP proteins, include: (1) LBP-1 and LBP-2; (2) LBP-1 and LBP-3; (3) LBP-2 and LBP-3; and (4) LBP-1, LBP-2, and LBP-3. This method optionally includes a step of diagnosing the subject as being at risk for atherosclerosis.

Also provided by the invention is a method of treating a subject at risk for atherosclerosis whereby a non-autologous LBP protein or a nucleic acid encoding a non-autologous LBP protein is delivered to the subject to generate an immune response to an autologous LBP. Specifically, this method entails identifying one or more autologous LBP proteins, e.g., LBP-1, LBP-2, or LBP-3, produced by the subject. The identification can by, e.g., DNA sequence analysis, protein sequence analysis, antibody reactivity, hybridization analysis, or nucleic acid amplification. Next, a non-autologous LBP protein, e.g., allogeneic, xenogeneic, or a genetically modified, non-naturally occurring protein that differs at one or more amino acid residues from the one or more LBP proteins, is administered to the subject. Alternatively, a nucleic acid encoding a non-autologous LBP protein is administered to the subject. The anti-atherosclerotic effectiveness of this immunotherapeutic product is determined by its ability to induce an immune response against one or more autologous LBP proteins when administered to the subject. It is therefore expected that extensive differences between a non-autologous and autologous LBP protein will not result in cross immunoreactivity. This method optionally includes a step of diagnosing the subject as being at risk for atherosclerosis.

Another method of the invention is a method of treating a subject at risk for atherosclerosis by increasing the levels of one or more LBP proteins circulating in the plasma. According to this method, either autologous or non-autologous LBP levels may be increased. Non-autologous LBP proteins include, e.g., allogeneic LBP, xenogeneic LBP, and genetically modified LBP. The plasma levels of one or more LBP proteins can be increased by the delivery of a nucleic acid encoding an LBP protein. Because LBP generally does not normally occur as a circulating protein, the endogenous molecule is expected to be susceptible to immune recognition when delivered in a soluble form. This method optionally includes a step of diagnosing the subject as being at risk for atherosclerosis.

Also included in the invention is a pharmaceutical composition containing one or more LBP proteins, e.g., LBP-1, LBP-2, or LBP-3, mixed with an adjuvant, suitable for use in humans. The pharmaceutical composition can contain a combination of more than one LBP protein. For example, compositions can include any of the following: (1) LBP-1 and LBP-2; (2) LBP-1 and LBP-3; (3) LBP-2 and LBP-3; and (4) LBP-1, LBP-2, and LBP-3.

Also included in the invention is a cell therapy system, whereby a cell expressing an LBP is delivered to a subject at risk for atherosclerosis. This cell can be engineered to express either an autologous or non-autologous LBP protein or peptide of the invention. Delivery of this engineered cell to a subject results in the in vivo production of an LBP protein and the associated immunotherapy produced when either the protein or a nucleic acid encoding the protein is provided to an individual. Cell therapy methods are described in U.S. 5,955,095 (herein incorporated by reference).

The following non-limiting examples further illustrate the present invention.

EXAMPLES

Example 1: Construction of a Rabbit cDNA Library

This example illustrates the construction of a rabbit cDNA library using mRNA from balloon-deendothelialized healing rabbit abdominal aorta. Balloon-catheter deendothelialized rabbit aorta has been shown to be a valid model for atherosclerosis

(Minick et al., Am. J. Pathol. 95:131-158 (1979).

The mRNA was obtained four weeks after ballooning to maximize focal LDL binding in the ballooned rabbit aorta. First strand cDNA synthesis was carried out in a 50 µl reaction mixture containing 4 µg mRNA; 2 µg oligo d(T)primer; methylation dNTP mix (10 mM each); 10 mM DTT; 800 units superscript II RT (Life Technologies, Gaithersburg, MD); 1 X first strand cDNA synthesis buffer (50 mM Tris-HCl, pH 8.3; 75 mM KCl; 5 mM $MgCl_2$), which was incubated for 1 hr at 37°C. The reaction mixture was then adjusted to 250 µl through the addition of 1 X second strand buffer (30 mM Tris-HCl, pH 7.5; 105 mM KCl; 5.2 mM $MgCl_2$); 0.1 mM DTT; methylation dNTP mix (10 mM each); 50 units E. coli DNA polymerase I, 3 units RNase H; 15 units E. coli DNA ligase (all enzymes from Life Technologies), which was incubated for an additional 2.5 hr at 15°C. The resulting double-stranded cDNAs (dscDNA) were then treated with 1.5 units T4 DNA polymerase (Novagen Inc., Madison, WI) for 20 min at 11°C to make blunt-ended dscDNA. These were then concentrated by ethanol precipitation and EcoRI/Hind III linkers were attached to the ends by T4 DNA ligase (Novagen Inc.). The linker-ligated cDNAs were treated with EcoRI and Hind111 restriction enzymes to produce EcoRI and Hind III recognition sequences at their 5'and 3'ends, respectively. After the removal of linker DNA by gel exclusion chromatography, the dscDNAs were inserted into λEXlox phage arms (Novagen Inc.) in a unidirectional manner by T4 DNA ligase and packaged into phage particles according to the manufacturer's protocol (Novagen Inc.). A phage library of cDNAs containing 2 x 10^6 independent clones was established from 4 µg of mRNA.

Example 2: Identification of Rabbit cDNAs Encoding LDL Binding Proteins (LBPs)

This example illustrates a method of functionally screening a rabbit cDNA library so as to identify cDNAs encoding LBPs which bind to both native LDL and methyl LDL. Methyl LDL is not recognized by previously reported cell surface receptors. See, e.g., Weisgraber et al., J. Biol. Chem. 253:9053-9062 (1978).

A fresh overnight culture of E. coli ER1647 cells (Novagen Inc.) was infected with the cDNA phage obtained from Example 1, and plated at a density of 2 x 10^4 plaque-forming units (pfu) in 150 mm diameter plates containing 2 X YT agar. A total of

50 plates, equivalent to 1 x 10^6 phage, were plated and incubated at 37°C until the plaques reached 1 mm in diameter (5-6 hr). A dry nitrocellulose membrane, which had previously been saturated with 10 mM IPTG solution, was layered on top of each plate to induce the production of recombinant protein, as well as to immobilize the proteins on the membranes. The plates were incubated at 37°C for an additional 3-4 hr, and then overnight at 4°C.

The next day, the membranes were lifted from each plate and processed as follows. Several brief rinses in TBST solution (10 mM Tris-HCl, pH 8.0; 150mM NaCl, 0.05% Tween 20); two 10-min rinses with 6M guanidine-HCl in HBB (20mM HEPES, pH 7.5; 5mM $MgCl_2$, 1mM DTT, and 5mM KCl); two 5-min rinses in 3M guanidine-HCl in HBB; a final brief rinse in TBSEN (TBS, 1mM EDTA, 0.02% NaN_3).

The membranes were then blocked for 30 min at room temperature in a solution of TBSEN with 5% non-fat dry milk, followed by 10 min in TBSEN with 1% non-fat dry milk. Following blocking, the membranes were incubated with native human LDL (obtained as described in Example 11 or methylated human LDL (meLDL) (see Weisgraber et al., J. Biol. Chem. 253:9053-9062 (1978)), at a concentration of 4 μg/ml, in a solution containing 1 X TBSEN, 1% non-fat dry milk, 1mM PMSF, 0.5 X protease inhibitor solution (1mM ε-amino caproic acid/lmM benzamidine). Incubation was for 4 hr at room temperature in a glass Petri dish with gentle stirring on a stirring table, followed by overnight at 4°C with no stirring.

Specifically bound meLDL and native LDL were detected on the nitrocellulose membranes by antibodies against human LDL. Sheep anti-human LDL polyclonal antibodies (Boehringer Mannheim, Indianapolis, IN) were adsorbed with E. coli plys E cell extracts to abolish background. For adsorption, E. coli plys E cells were grown to log phase, spun down and resuspended in PBS containing 1 mM PMSF, 2 mM ε-amino caproic acid, and 1 mM benzamidine. The cell suspension then underwent 8 freeze-thaw cycles via immersion in liquid nitrogen and cold running tap water, respectively. The anti LDL antibodies/cell extract solution were incubated with gentle stirring for 1 hr at 4°C (1 ml of antibody solution/3 mg crude cell extract). Following incubation, the mixture was centrifuged (10,000 x g; 10 min; 4°C) and the supernatant was stored at 4°C in the presence of 0.02% NaN_3, until use. The membranes were processed for immunoscreening

as follows: (i) three 5-min washes at room temperature in TBSEN containing 1% gelatin; (ii) 30 min incubation in PBS, pH 7.4 with 1% gelatin; (iii) two-hr room temperature incubation with gentle stirring in fresh PBS/gelatin solution containing adsorbed sheep anti-human LDL antibodies (Boehringer Manheim, Indianapolis, IN) (1: 1000 dilution); (iv) three brief washes in TBS, pH 7.4; (v) one-hr room temperature incubation with gentle stirring in PBS/gelatin solution containing donkey anti-sheep alkaline phosphatase-conjugated antibodies (Sigma, St. Louis, MO) (1: 10,000 dilution); (vi) three brief washes with TBS, PH 7.4.; and (vii) development according to the manufacturer's instructions, using an alkaline phosphatase substrate development kit (Novagen Inc.). Phage plaques which produced LBPs appeared as blue-colored "donuts" on the membranes.

The phage from Example 1 containing the LBP cDNAs were plaque-purified and converted into plasmid subclones by following a protocol called "Autosubcloning by Cre-mediated Plasmid Excision" provided by Novagen Inc. DNA sequences were obtained by the dideoxynucleotide chain-termination method (Sanger et al., Proc. Natl. Acad. Sci., USA 74: 5463-5467 (1977), and analyzed by an Applied Biosystems automated sequencer. The open reading frame (ORF) of each cDNA was determined from consensus sequences obtained from both the sense and antisense strands of the cDNAs. Sequencing confirmed that three previously unknown genes had been isolated. Since the genes were selected by functional screening for LDL binding, the proteins coded by these genes were termed LDL binding proteins (LBPs), specifically, LBP-1, LBP-2 and LBP-3. The cDNA sequences for rabbit LBP-1, LBP-2 and LBP-3 and the corresponding proteins are set forth in SEQ ID NOS: 10-14 and 48.

Based on their respective cDNA coding sequences, the sizes of the recombinant proteins were determined to be 16.2 kDa for LBP-1, 40 kDa for LBP-2, and 62.7 kDa for LBP-3.

Example 3: Northern Blot Analysis of Rabbit RNA Using LBP cDNA or cRNA

This example illustrates the size and tissue distribution of LBP mRNAs. Total RNA was isolated from different rabbit tissues: adrenals, thoracic aorta, abdominal aorta, ballooned and reendothelialized abdominal aorta, heart, kidney, smooth muscle cells,

lung and liver, by Trizol reagent (Life Technologies) and concentrated by ethanol precipitation. Gel electrophoresis of RNA was carried out in 1.2% agarose gel containing 1 X MOPS buffer (0.2M MOPS, pH 7.0; 50 mM sodium acetate; 5mM EDTA, pH 8.0) and 0.37M formaldehyde. Gels were loaded with 20 μg total RNA from each tissue examined and electrophoresed at 100 volts for 2 hr in 1 X MOPS buffer. RNAs were blotted onto supported nitrocellulose membranes (Schleicher & Schuell, Keene, NH) and immobilized by baking at 80°C for 2 hr. Hybridization to radiolabeled LBP-1, LBP-2 and LBP-3 cDNA or cRNA probes was carried out by standard procedures known to those skilled in the art (see, e.g., Ausubel et al., Current Protocols in Molecular Biology; John Wiley & Sons (1989)); signals were detected by autoradiography.

The results were as follows: the sizes of the mRNAs were about 1.3 kb for LBP-1, about 2.3-2.5 kb for LBP-2, and about 4.7 kb for LBP-3. LBP-1, LBP-2 and LBP-3 mRNA were found in all tissues tested, but the highest amount was in ballooned abdominal aorta.

Example 4: Isolation of Human LBP cDNAs and Genomic Clones

This example illustrates isolation of human LBP cDNAs. Human LBP cDNA clones were isolated from three cDNA libraries. A human fetal brain cDNA library was obtained from Stratagene, LaJolla, CA, a human liver and a human aorta cDNA library were obtained from Clontech, Palo Alto, CA, and screened with a radiolabeled cDNA probe derived from rabbit LBP-1, LBP-2 or LBP-3, according to the method described in Law et al., Gene Expression 4:77-84 (1994). Several strongly hybridizing clones were identified and plaque-purified. Clones were confirmed to be human LBP-1, LBP-2 and LBP-3, by DNA sequencing using the dideoxynucleotide chain-termination method and analysis by an Applied Biosystems automated sequencer. The cDNA sequences and the corresponding proteins for human LBP-1, LBP-2 and LBP-3 are set forth in SEQ ID NOS: 15, 16 and 17, respectively.

A human genomic library was screened with each of the LBP-1, LBP-2, and LBP-3 clones obtained from the cDNA library screening. Clones hybridizing to each of the three cDNAs were isolated and sequenced. The genomic sequence for LBP-1, LBP-2, and LBP-3 are set forth in Figs. 22-24, respectively. The LBP-1 open reading frame

spans four exons of the LBP-1 gene (Fig. 22; SEQ ID NO:49). The LBP-1 protein predicted by the genomic sequence is identical to that predicted by the cDNA clone described above. The LBP-2 open reading frame spans five exons of the LBP-2 gene (Fig. 23; SEQ ID NO:50). The LBP-2 protein predicted by the genomic sequence differs from that predicted by the cDNA clone in that it contains an additional 321 amino acids at its amino terminus (the LBP-2 cDNA is a 5' truncation). The LBP-3 open reading frame spans ten exons of the LBP-3 gene (Fig. 24; SEQ ID NO:51). The LBP-3 protein predicted by the genomic sequence differs from that predicted by the cDNA clone in that it contains an additional 16 amino acids at its amino terminus (the LBP-3 cDNA is a 5' truncation) and an Asn at amino acid position 130 (the cDNA predicts a Tyr at this position). A comparison between the corresponding LBP-1, LBP-2 and LBP-3 protein sequences for rabbit and human are shown in Figs. 19, 20 and 21.

Example 5: Isolation of Recombinant LBP-1, LBP-2 and LBP-3 Rabbit Proteins from E. coli

LBP cDNA was isolated from the original pEXlox plasmids obtained as described in Examples 1 and 2, and subcloned into the pPROEX-HT vector (Life Technologies) for recombinant protein expression. Induction of the recombinant protein by IPTG addition to transformed E. coli DH10B cultures resulted in the expression of recombinant protein containing a 6-histidine tag (N-terminal). This tagged protein was then purified from whole cell proteins by binding to Ni-NTA (nickel nitrilo-triacetic acid) as described in the protocol provided by the manufacturer (Qiagen, Inc., Santa Clara, CA). The preparation obtained after the chromatography step was approximately 90% pure; preparative SDS-PAGE was performed as the final purification step.

When required by the characterization procedure, iodination of LBPs was carried out using Iodobeads (Pierce, Rockford, IL). The Iodobeads were incubated with 500 μCi of $Na^{125}I$ solution (17 Ci/mg) (New England Nuclear, Boston, MA) in a capped microfuge tube for 5 min at room temperature. The protein solution was added to the Iodobeads-$Na^{125}I$ microfuge tube and incubated for 15 min at room temperature. At the end of this incubation, aliquots were removed for the determination of total soluble and TCA precipitable counts. The radiolabeled protein was then precipitated with cold

acetone (2.5 vol; -20°C; 2.5 hr). Following this incubation, precipitated protein was collected by centrifugation (14,000 g; 1 hr; room temperature) and resuspended in sample buffer (6 M urea/50 mM Tris, pH 8.0/2 mM EDTA). Integrity of the protein preparation was assessed by SDS-PAGE.

The identities of the recombinant LBPs were confirmed using standard protein sequencing protocols known to those skilled in the art. (A Practical Guide for Protein and Peptide Purification for Microsequencing, Matsudaira, ed., Academic Press, Inc., 2d edition (1993)). Analysis was performed using an Applied Biosystems Model 477A Protein Sequencer with on-line Model 120 PTH amino acid analyzer.

Example 6: Production of Antibodies to LBP-1, LBP-2 and LBP-3

This example illustrates the production of polyclonal antibodies to LBP-1, LBP-2 and LBP-3. A mixture of purified recombinant LBP protein (0.5 ml; 200 μg) and RIBI adjuvant (RIB1 ImmunoChem. Research, Inc., Hamilton, MT) was injected subcutaneously into male guinea pigs (Dunkin Hartley; Hazelton Research Products, Inc., Denver, PA) at 3-5 sites along the dorsal thoracic and abdominal regions of the guinea pig. Blood was collected by venipuncture on days 1 (pre-immune bleeding), 28, 49 and 70. Booster injections were administered on days 21 (100 μg; SC), 42 (50 μg; SC), and 63 (25 μg; SC). The titer of the guinea pig antiserum was evaluated by serial dilution "dot blotting." Preimmune antiserum was evaluated at the same time. After the third booster of LBP protein, the titer against the recombinant protein reached a maximal level with a detectable calorimetric response on a dot blot assay of 156 pg.

Specificity of the polyclonal antibody for recombinant LBP-1, LBP-2 or LBP-3 was demonstrated using Western blot analysis. (Towbin et al., Proc. Natl. Acad. Sci. USA 76: 4350 (1979)). The protein-antibody complex was visualized immunochemically with alkaline phosphatase-conjugated goat antiguinea pig IgG, followed by staining with nitro blue tetrazolium (BioRad Laboratories, Hercules, CA). Non-specific binding was blocked using 3% non-fat dry milk in Tris buffered saline (100 mM Tris; 0.9% NaCl, pH 7.4).

Example 7: Immunohistochemical Characterization

This example illustrates the presence of LBPs in or on endothelial cells covering plaques, in or on adjacent smooth muscle cells, and in the extracellular matrix. In addition, co-localization of LDL and LBPs was demonstrated. These results were obtained by examining ballooned rabbit arterial lesions and human atherosclerotic plaques by immunohistochemical methods.

Ballooned deendothelialized aorta was obtained from rabbits which had received a bolus injection of human LDL (3 mg; i.v.) 24 hr prior to tissue collection. Human aortas containing atherosclerotic plaques were obtained from routine autopsy specimens. Tissues were fixed in 10% buffered formalin (≤24 hr) and imbedded in paraffin using an automated tissue-imbedding machine. Tissue sections were cut (5-7 μ) and mounted onto glass slides by incubating for 1 hr at 60°C. Sections were deparaffinized. After a final wash with deionized H_2O, endogenous peroxidase activity was eliminated by incubating the sections with 1% H_2O_2/H_2O buffer for 5 min at room temperature. Sections were rinsed with phosphate buffered saline (PBS) for 5 min at room temperature and nonspecific binding was blocked with 5% normal goat serum or 5% normal rabbit serum depending on the source of the secondary antibody (Sigma, St. Louis, MO) (1 hr; room temperature). Sections were then incubated with a 1:50 dilution (in 5% normal goat serum/PBS) of a guinea pig polyclonal antibody against the rabbit form of recombinant LBP-1, LBP-2 or LBP-3. Controls included preimmune serum as well as specific antisera to LBP-1, LBP-2, or LBP-3 in which the primary antibody was completely adsorbed and removed by incubation with recombinant LBP-1, LBP-2 or LBP-3 followed by centrifugation prior to incubation with the tissue sections. An affinity purified rabbit polyclonal antibody against human apolipoprotein B (Polysciences Inc.; Warrington, PA) was used at a dilution of 1:100 (in 5% normal rabbit serum/PBS). Sections were incubated for 2 hr at room temperature in a humidified chamber. At the end of incubation, sections were rinsed with PBS and incubated with a 1:200 dilution (in 5% normal goat serum/PBS) of goat anti-guinea pig biotinylated IgG conjugate (Vector Laboratories, Burlingame, CA) or a 1:250 dilution (in 5% normal rabbit serum/PBS)of rabbit anti-goat biotinylated IgG conjugate (Vector Laboratories, Burlingame, CA) for 1 hr at room temperature in a humidified chamber. Sections were then rinsed with PBS and antigen-antibody signal

amplified using avidin/biotin HRP conjugate (Vectastain ABC kit; Vector Laboratories, Burlingame, CA). Sections were developed using DAB substrate (4-6 min; room temperature) and counterstained with hematoxylin. In the ballooned rabbit artery, immunohistochemistry with the anti-LBP-1, LBP-2 and LBP-3 antibodies showed that LBP-1, LBP-2 and LBP-3 were located in or on functionally modified endothelial cells at the edges of regenerating endothelial islands, the same location in which irreversible LDL binding has been demonstrated (Chang et al., Arteriosclerosis and Thrombosis 12:1088-1098 (1992)). LBP-1, LBP-2 and LBP-3 were also found in or on intimal smooth muscle cells underneath the functionally modified endothelial cells, and to a lesser extent, in extracellular matrix. No LBP-1, LBP-2 or LBP-3 was detected in still deendothelialized areas, where LDL binding had been shown to be reversible (Chang et al., Arteriosclerosis and Thrombosis 12:1088-1098 (1992)). Immunohistochemistry of ballooned rabbit aorta with anti-human apolipoprotein B antibodies showed the presence of LDL at the same locations as that found for LBP-1, LBP-2 and LBP-3.

In the human atherosclerotic plaques taken at routine autopsies, immunohistochemistry with the anti-LBP-1,. anti-LBP-2 and anti-LBP-3 antibodies showed that LBP-1, LBP-2, and LBP-3 were also found in or on endothelial cells covering plaques and in or on adjacent smooth muscle cells. In the human tissue, there was greater evidence of LBP-1, LBP-2 and LBP-3 in extracellular matrix.

The results obtained with paraffin sections were identical to those of frozen sections.

Example 8: Affinity Coelectrophoresis (ACE) Assays of LBPs and LDL or HDL

This example illustrates that binding occurs between LBP-1, LBP-2 or LBP-3 and LDL, and that this binding is specific, as illustrated by the fact that binding does not occur between LBP-1, LBP-2 or LBP-3 and HDL (high density lipoprotein). Analysis of the affinity and specificity of recombinant rabbit LBP-1, LBP-2 or LBP-3 binding to LDL was carried out using the principle of affinity electrophoresis (Lee and Lander, Proc. Natl. Acad. Sci. USA 88:2768-2772 (1991)). Melted agarose (1%; 65°C) was prepared in 50 mM sodium MOPS, pH 7.0; 125 mM sodium acetate, 0.5% CHAPS. A teflon comb consisting of nine parallel bars (45 x 4 x 4 mm/3 mm spacing between bars)

was placed onto GelBond film (FMC Bioproducts, Rockland, ME) fitted to a plexiglass casting tray with the long axis of the bars parallel to the long axis of the casting tray. A teflon strip (66 x 1 x 1 mm) was placed on edge with the long axis parallel to the short axis of the casting tray, at a distance of 4 mm from the edge of the teflon comb. Melted agarose (>65°C) was then poured to achieve a height of approximately 4 mm. Removal of the comb and strip resulted in a gel containing nine 45 x 4 x 4 mm rectangular wells adjacent to a 66 x 1 mm slot. LDL or HDL samples were prepared in gel buffer (50mM sodium MOPS, pH 7.0, 125 mM sodium acetate) at twice the desired concentration. Samples were then mixed with an equal volume of melted agarose (in 50 mM MOPS, pH 7.0; 125 mM sodium acetate; 50°C), pipetted into the appropriate rectangular wells and allowed to gel. The binding affinity and specificity of LBP-1 and LBP-3 was tested using several concentrations of LDL (540 to 14 nM) and HDL (2840177 nM). A constant amount (0.003 nM -0.016 nM) of ^{125}I-labeled LBP-1, LBP-2 or LBP-3 (suspended in 50 mM sodium MOPS, pH 7.0; 125 mM sodium acetate; 0.5% bromphenol blue; 6% (wt/vol) sucrose) was loaded into the slot. Gels were electrophoresed at 70v/2hr/20°C. At the end of the run, the gels were air dried and retardation profiles were visualized by exposure of X-ray films to the gels overnight at -70°C, with intensifying screens.

LDL retarded LBP-1, LBP-2 and LBP-3 migration through the gel in a concentration-dependent, saturable manner, indicating that LBP-1, LBP-2 and LBP-3 binding to LDL was highly specific. This conclusion is supported by the fact that HDL did not retard LBP-1, LBP-2 or LBP-3. A binding curve generated from the affinity coelectrophoresis assay indicated that LBP-1 binds to LDL with a K_d of 25.6 nM, that LBP-2 (rabbit clone 26) binds to LDL with a K_d of 100 nM, and that LBP-3 (80 kDa fragment) binds to LDL with a K_d of 333 nM.

In addition to testing affinity and specificity of LBP-1, LBP-2 and LBP-3 binding to LDL, the ability of "cold" (i.e., non-radiolabeled) LBP-1, LBP-2 or LBP-3 to competitively inhibit radiolabeled LBP-1, LBP-2 or LBP-3 binding to LDL, respectively, was tested. Competition studies were carried out using fixed concentrations of cold LDL and radiolabeled LBP-1 and increasing amounts of cold recombinant LBP-1 (6-31 μM). The ACE assay samples and gel were prepared as described herein. Cold LBP-1 inhibited binding of radiolabeled LBP-1 to LDL in a concentration-dependent manner, cold LBP-2

inhibited binding of radiolabeled LBP-2 to LDL in a concentration-dependent manner, and cold LBP-3 inhibited binding of radiolabeled LBP-3 to LDL in a concentration-dependent manner.

Rabbit and human LBP-2 contain a long stretch of acidic amino acids at the amino terminal (rabbit LBP-2 amino acid residues 338 through 365 and human LBP-2 amino acid residues 329 through 354). The possibility that this segment of LBP-2 was the LDL binding domain was tested by subcloning two rabbit LBP-2 clones which differ from each other by the presence or absence of this acidic region (clone 26 and clone 45, respectively) into expression vectors, by standard methods known to those skilled in the art. ACE assays were then conducted in order to assess the affinity and specificity of the binding of these two clones to LDL. LDL retarded clone 26 derived radiolabeled LBP-2 migration through the gel in a concentration-dependent, saturable, manner while clone 45 derived radiolabeled LBP-2 migration was not retarded.

Competition studies using fixed concentrations of cold LDL and clone 26 derived radiolabeled LBP-2 and increasing concentrations of cold recombinant LBP-2/clone 26 and LBP-a/clone 45 were carried out. Cold clone 26 derived LBP-2 inhibited binding of clone 26 derived radiolabeled LBP-2 to LDL in a concentration-dependent manner. Clone 45 derived LBP-2, on the other hand, did not affect the binding of clone 26 derived radiolabeled LBP-2 to LDL. These results indicate that the long stretch of acidic amino acids contain a binding domain of LBP-2 to LDL.

Example 9: Affinity Coelectrophoreses (ACE) Assays of LBP-1 or LBP-2 and LDL in the Presence of Inhibitors

This example illustrates that binding between LBP-1 or LBP-2 and LDL is inhibited by polyglutamic acid or BHF-1. The ability of a third compound to inhibit binding between two proteins previously shown to interact was tested by a modification of the ACE assays described in Example 8. The third compound was added to the top or wells together with the radiolabeled protein. If the third compound inhibited binding, the radiolabeled protein would run through the gel. If the third compound did not inhibit binding, migration of the radiolabeled protein was retarded by the protein cast into the gel.

Inhibition of LBP-1/LDL or LBP-2/LDL binding by polyglutamic acid (average MW about 7500, corresponding to about 7 monomers) was shown by casting a constant amount of LDL (148 nM) in all the rectangular lanes. A constant amount (1 µl) of ^{125}I-labeled LBP-1 or LBP-2 (0.003 nM -0.016 nM) was loaded in the wells at the top of the gel, together with increasing concentrations of polyglutamic acid (obtained from Sigma) (0-0.4 nM). The gel was electrophoresed at 70 volts for 2 hr, dried and placed on X-ray film, with intensifying screens, overnight at -70°C before the film was developed to determine the retardation profile of LBP-1 and LBP-2. As the concentration of polyglutamic acid increased, retardation of radiolabeled LBP-1 and LBP-2 migration by LDL decreased in a concentration-dependent manner, which showed that polyglutamic acid inhibited binding between LBP-1, LBP-2 and LDL.

Inhibition of LBP-1/LDL binding by BHF-1 was shown by casting a constant amount of LDL (148 nM) in all the rectangular lanes. A constant amount of ^{125}I-labeled LBP-1 (0.003 nM -0.016 nM) was loaded in the wells at the top of the gel, together with increasing concentrations of BHF-1 (0-10 nM), obtained as described in Example 15. The gel was electrophoresed at 70 volts for 2 hr, dried and placed on X-ray film, with intensifying screens, overnight at -70°C. The film was then developed to determine the retardation profile of ^{125}I-LBP-1. As the concentration of BHF-1 increased, retardation of LBP-1 by LDL decreased in a concentration-dependent manner, which demonstrated that BHF-1 inhibited binding between LBP-1 and LDL.

Example 10: Affinity Coelectrophoreses (ACE) Assays for Identifying Fragments, Analogs and Mimetics of LBPs which Bind to LDL

This example illustrates a method for identifying fragments, analogs or mimetics of LBPs which bind to LDL, and which thus can be used as inhibitors of LDL binding to LBP in the arterial walls, by occupying binding sites on LDL molecules, thereby rendering these sites unavailable for binding to LBP in the arterial wall.

Fragments of LBPs are generated by chemical cleavage or synthesized from the known amino acid sequences. Samples of these fragments are individually added (cold) to radiolabeled LBP as described in Example 8, to assess the inhibitory potency of the various fragments. By iterative application of this procedure on progressively smaller

portions of fragments identified as inhibitory, the smallest active polypeptide fragment or fragments are identified. In a similar manner, analogs of the LBPs are tested to identify analogs which can act as inhibitors by binding to LDL. And, similarly, mimetics of LBP (molecules which resemble the conformation and/or charge distributions of the LDL-binding sites on LBP molecules) are tested in a similar fashion to identify molecules exhibiting affinities for the LDL-binding sites on LBP.

The affinities of the inhibitors so identified are at least as strong as the affinity of LDL itself for the LDL-binding sites on LBP. The inhibitors bind at least competitively, and some irreversibly and preferentially as well, to the LDL-binding sites, thereby rendering such sites unavailable for binding to humoral LDL.

Example 11: ELISA Assays

This example illustrates the use of ELISA plate assays for the quantification of a test compound's capacity to inhibit the binding of LDL to a specific LBP.

In one example, the ELISA assay was carried out as follows: LDL was diluted in 50 mM Na_2HCO_3, pH 9.6/0.02% NaN_3 and added to the wells of a 96-well plate (ImmunoWare 96-Well Reacti-Bind EIA Polystyrene Plates; Pierce (Rockford, IL)) to achieve a final concentration ranging from 0.1 to 1 μg/well. The plates were incubated for 6 hr at room temperature. At the end of the incubation period, the wells were washed 3 times with Tris-buffered saline, pH 7.4 (TBS), and blocked overnight with 200 μl of 1% bovine serum albumin (BSA) in TBS/0.02% NaN_3 (Sigma; St. Louis MO) at room temperature. The wells were then incubated with 200 μl of LBP protein (5-10 μg/well) in TBS and varying concentrations of the test compound. Plates were incubated for 1 hr at room temperature. The wells were then washed three times with TBS and blocked for 2 hr with 200 μl of 1% BSA in TBS/0.02% NaN_3 at room temperature. At the end of the incubation period, the wells were washed 3 times with TBS and a 1: 1000 dilution (in TBS/0.05% Tween 20) of the appropriate guinea pig anti-LBP protein polyclonal antibody was added to the wells and incubated for 1 hr at room temperature. The wells were then washed 3 times with TBS/0.05% Tween 20; a 1:30,000 dilution of goat anti-guinea pig IgG alkaline phophatase conjugate (Sigma) was added to each well. Plates were incubated for 1 hr at room temperature. The wells were washed 3 times with

TBS/0.05% Tween 20 and a calorimetric reaction was carried out by adding 200 ml of p-nitrophenyl phosphate substrate (Sigma; St. Louis MO) to the wells. The reaction was allowed to proceed for 30 min at room temperature and stopped with 50µl of 3N NaOH. The absorbance was determined at 405 nm using an ELISA plate reader. The test compound's effectiveness in blocking the binding of LDL to the recombinant protein was assessed by comparing the absorbance values of control and treated groups.

In a second example, the ELISA assay was carried out as follows: LDL was diluted in Tris-buffered saline, pH 7.4 (TBS) and added to the wells of a 96-well plate (ImmunoWare 96-Well Reacti-Bind EIA Polystyrene Plates; Pierce (Rockford, IL)) to give a plate-saturating concentration of 0.2 µg/well. The plate was incubated for 1 hr at room temperature, after which the wells were washed three times with TBS, before being blocked for 1 hr at room temperature with 1% bovine serum albumin (BSA in TBS). The wells were then washed twice with TBS before LBP-1 or LBP-2 (0.025 µg/well), or LBP-3 (0.01 µg/well) were added, without and with varying concentrations of the test inhibitor compound. Each condition was set up in quadruplicate. The plate was incubated for 1 hr at room temperature, then washed three times with TBS/0.02% Tween 20 (TBS/Tween). An appropriate dilution of guinea pig anti-LBP polyclonal antibody (1:750 to 1:1500, depending on the antibody) was added to three wells for each condition and incubated for 1 hr. Anti-LBP antibody was replaced by buffer for the fourth well of each condition, as a negative control. After 1 hr, the plate was again washed three times with TBS/Tween before a 1:10,000 dilution (in TBS/Tween) of goat anti-guinea pig IgG alkaline phosphatase-conjugated antibody (Sigma) was added to each well. The plate was incubated for 1 hr at room temperature, then washed three times with TBS/Tween. A fresh solution of substrate was prepared from an Alkaline Phosphatase Substrate Kit (Bio-Rad, Hercules, CA) as follows: Mix 1 ml 5x concentrated diethanolamine buffer with 4 ml distilled water. Add one tablet of p-nitrophenylphosphate (5 mg) and vortex until tablet is completely dissolved. Subtrate solution was added to wells immediately. Increasing concentrations of diluted alkaline phosphatase-conjugated goat anti-guinea pig IgG (1: 100,000 dilution in TBS/Tween) were added to five empty wells, followed by substrate, as a positive control. Following addition of substrate, the plate was immediately placed in an ELISA plate reader, allowed to stand at 37°C, generally for 75

min, before absorbance was measured at 405 nm. Incubation in the ELISA reader at 37°C was sometimes adjusted to optimize absorbance (60-90 min). The effectiveness of the test inhibitor was determined, after subtracting absorbance of negative controls, by comparing absorbance in wells where an LBP was mixed with test inhibitor to absorbance in wells containing LBP with no inhibitor.

Alternatively, LBPs, rather than LDL, were bound to the plate. Recombinant LBP protein binding to LDL and the effect of varying concentration of the inhibitor on LBP-LDL binding was determined through the use of antibodies against LDL. This interaction was visualized through the use of a secondary antibody conjugated to a reporter enzyme (e.g. alkaline phosphatase).

ELISA plate assays were used to screen agents which can affect the binding of LBP proteins to LDL. For example, peptides derived from LBP-1 and human LBP-3 protein sequences (BHF-1 and BHF-2, respectively) were synthesized and have been shown to reduce the binding of LDL to recombinant LBP-1 and LBP-2 in this format. These results were in agreement with those obtained with the ACE assays.

Example 12: Administration of Humanized Antibodies Against LBPs so as to Block LDL-Binding Sites on the LBPs

This example illustrates administration to patients of humanized antibodies against LBP-1, LBP-2 or LBP-3 so as to block LDL-binding sites on arterial LBP molecules. Mouse monoclonal antibodies are humanized by recombinant DNA techniques and produced by standard procedures known to those skilled in the art (Berkower, I., Curr. Opin. Biotechnol. 7:622-628 (1996); Ramharayan and Skaletsky, Am. Biotechnol. Lab 13: 26-28 (1995)) against LBPs and/or the LDL-binding sites on the LBPs. The corresponding Fab fragments are also produced, as described in Goding, J. W., Monoclonal Antibodies: Principles and Practice, Academic Press, New York, NY (1986). These antibodies are administered parenterally in sufficient quantity so as to block LDL-binding sites on the LBP molecules, i.e., 1-10 mg/kg daily. This prevents the irreversible arterial uptake of LDL that is required to facilitate oxidation of the LDL.

Example 13: Preparation of LDL

This example illustrates the preparation of LDL. LDL was prepared from the plasma of normolipemic donors (Chang et al., Arterioscler. Thromb. 12:1088-1098 (1992)). 100 ml of whole blood was placed into tubes containing 100 mM disodium EDTA. Plasma was separated from red blood cells by low-speed centrifugation (2,000 g; 30 min; 4°C). Plasma density was adjusted to 1.025 gm/ml with a solution of KBr and centrifuged for 18-20 hr, 100,000 x g, 12°C. Very low density lipoproteins (VLDL) were removed from the tops of the centrifuge tubes with a Pasteur pipette. The density of the infranate was raised to 1.050 gm/ml with KBr solution and centrifuged for 22-24 hr, 100,000 x g, 12°C. LDL was removed from the tops of the centrifuge tubes with a drawn out Pasteur pipette tip. Purity of the LDL preparation was checked by Ouchterlony double immunodiffusion against antibodies to human LDL, human HDL, human immunoglobulins, and human albumin. KBr was removed from the LDL solution by dialysis (1L, x 2, approximately 16 hr) against 0.9% saline, pH 9.0, containing 1 mM EDTA and 10 μM butylated hydroxytoluene (BHT), the latter to prevent oxidation of LDL. Following dialysis, LDL protein was measured by the method of Lowry (Lowry et al., J. Biol. Chem. 193:265-275 (1951)), and the LDL was stored at 4°C until use. LDL preparations were kept for no more than 4-6 weeks.

Example 14: Preparation of HDL

This example illustrates the preparation of HDL. HDL was prepared from plasma of normolipemic donors. 100 ml of whole blood was placed into tubes containing 100 mM disodium EDTA and plasma was collected by centrifugation (2000 g; 30 min; 4°C). Apolipoprotein B containing lipoproteins present in plasma were then precipitated by the sequential addition of sodium heparin (5,000 units/ml) and $MnCl_2$ (1M) to achieve a final concentration of 200 units/ml and 0.46 M, respectively (Warnick and Albers, J. Lipid Res. 19:65-76 (1978)). Samples were then centrifuged, (2000 g; 1 hr; 4°C). The supernatant was collected and density adjusted to 1.21 g/ml by the slow addition of solid KBr. HDL was separated by ultracentrifugation (100,000 g; >46 hr; 12°C). Purity of the HDL preparation was assessed via Ouchterlony double immunodiffusion test using antibodies against human HDL, human LDL, human immunoglobulins, and human

albumin. HDL samples were dialyzed against saline pH 9.0/lmM EDTA/l0μM BHT (4L; 24 hr/4°C) and total protein was determined by the Lowry protein assay (Lowry et al., J. Biol. Chem. 193:265-275 (1951)). HDL was stored at 4°C until use. HDL preparations were kept for no longer than 2 weeks.

Example 15: Synthesis of BHF-1

This example illustrates the synthesis of BHF-1, a fragment of human or rabbit LBP-1 which contains amino acid residues 14 through 33. BHF-1 was synthesized using an Applied Biosystems Model 430A peptide synthesizer with standard T-Boc NMP chemistry cycles. The sequence of BHF-1 is as follows:

val-asp-val-asp-glu-tyr-asp-glu-asn-lys-phe-val-asp-glu-glu-asp-gly-gly-asp-gly (SEQ ID NO: 9).

After synthesis, the peptide was cleaved with hydrofluoric acid/anisole (10/l v/v) for 30 min at -10°C and then incubated for 30 min at 0°C. BHF-1 was then precipitated and washed three times with cold diethyl ether. Amino acid coupling was monitored with the ninhydrin test (>99%).

The BHF-1 peptide was purified to homogeneity by high performance liquid chromatography on a reverse phase Vydac C_4 column (2.24 X 25 cm) using a linear gradient separation (2-98%B in 60 min) with a flow rate of 9 ml/min. Buffer A consisted of 0.1% trifluoroacetic acid (TFA)/Milli Q water and Buffer B consisted of 0.085% TFA/80% acetonitrile. The gradient was run at room temperature and absorbance monitored at 210 and 277 nm.

Fast atom bombardment-mass spectrometry gave a protonated molecular ion peak $(M+H)^+$ at m/z= 2290.2, in good agreement with the calculated value. On amino acid analysis, experimental values for the relative abundance of each amino acid in the peptide were in good agreement with theoretical values. The lyophilized peptide was stored at -20°C.

Example 16: In Vitro Screening for Agents Which Inhibit Binding Between LDL and LBPs

This example illustrates in vitro screening for agents which inhibit binding

between LDL and LBPs.

A candidate polypeptide for being an agent is chosen, e.g., LBP-1, LBP-2, LBP-3, BHF-1 or any other polypeptide. The shortest fragment of the polypeptide that inhibits LDL binding to LBPs in vitro is determined. Peptides are synthesized by standard techniques described herein. Inhibition assays are performed using standard ELISA techniques for screening, and affinity coelectrophoresis (ACE) assays to confirm the ELISA results, as described herein. Additional assays that can be used in this screening method include, e .g., fluorescence polarization and pulsed ultra-filtration electrospray mass spectrometry. Short peptides ranging, e.g., from dimers to 20-mers are constructed across sequences of the candidate polypeptide whose chemical characteristics make them likely LDL binding sites, e.g., acidic regions. The ability of shorter and shorter lengths of the peptides to inhibit LDL binding in vitro and to mammalian cells in culture is tested. For example, the effect of the peptide on inhibiting LDL binding in mammalian cells transfected to express an LBP gene is tested. Each of the peptides so identified as an inhibitor is tested with each of LBP-1, LBP-2 and LBP-3, to determine whether a single inhibitor works against all three LBPs.

Once the minimum active sequence is determined, the peptide backbone is modified so as to inhibit proteolysis, as discussed herein. For example, modification is accomplished by substitution of a sulfoxide for the carbonyl, by reversing the peptide bond, by substituting a methylene for the carbonyl group, or other similar standard methodology. See Spatola, A.F., "Peptide Backbone Modifications: A Structure-Activity Analysis of Peptides Containing Amide Bond Surrogates, Conformational Constraints, and Related Backbone Replacements," in Chemistry and Biochemistry of Amino Acids, Peptides and Proteins, Vol. 7, pp. 267-357, B. Weinstein (ed.), Marcel Dekker, Inc., New York (1983). The ability of these analogs to inhibit LDL binding to the LBPs in vitro is tested in a similar manner as for the natural peptides described above, e.g., by ELISA, ACE, fluorescence polarization, and/or pulsed ultra-filtration electrospray mass spectrometry.

Example 17: In Vitro Screening With Cultured Mammalian Cells for Agents Which Inhibit Binding between LDL and LBPs

This example illustrates cell-based in vitro screening of agents which have been shown by in vitro tests such as ACE assay and ELISA to be potential inhibitors of binding between LDL and LBPs.

Mammalian cells, such as 293 cells, which are commonly used for expression of recombinant gene constructs, are used to develop cell lines which express LBPs on the cell surface. This is done by subcloning LBP open reading frames (ORFS) into a mammalian expression plasmid vector, pDisplay (Invitrogen, Carlsbad, CA), which is designed to express the gene of interest on the cell surface. The use of mammalian cells to produce LBPs allows for their expression in a functionally active, native conformation. Therefore, stably transfected mammalian cell lines with surface expression of LBPs individually, or in combination, are particularly suitable for assaying and screening inhibitors that block LDL binding in cell culture, as well as to evaluate the cytotoxicity of these compounds.

Specifically, LBP ORFs are amplified by PCR (Perkin Elmer, Foster City, CA) from cDNA templates using Taq polymerase (Perkin Elmer) and appropriate primers. The amplified LBP ORFs are purified by agarose gel electrophoresis and extracted from gel slices with the Bio-Rad DNA Purification kit (Bio-Rad, Hercules, CA). The purified DNAs are then cut with the restriction enzymes Bgl II and Sal I (New England Biolabs, Beverly, MA) to generate cohesive ends, and purified again by agarose gel electrophoresis and DNA extraction as described above. The LBP ORFs are then subcloned into the Bgl II/Sal I sites in the mammalian expression vector, pDisplay (Invitrogen) by ligation. Recombinant plasmids are established by transformation in E. coli strains TOP10 (Invitrogen) or DH5α (Life Technologies, Grand Island, NY). Recombinant pDisplay/LBP plasmid DNA is isolated from overnight E. coli cultures with the Bio-Rad Plasmid Miniprep kit, cut with Bgl II/Sal I, and analyzed by agarose gel electrophoresis. LBP ORFs in successfully transformed clones are verified by automated dideoxy DNA sequencing. To transfect human kidney 293 cells, 1-2 μg of DNA is mixed with 6 μl lipofectamine reagent (Life Technologies) and incubated with the cells as described in the Life Technologies protocol. LBP expression in transfected cells is confirmed by Western blot analysis of cell extracts obtained 48 hr after transfection. To select for stably transfected 293 cells, the antibiotic G418 (Life Technologies) is added to

the growth medium at a concentration of 800 μg/ml. Colonies resistant to G418 are tested for recombinant LBP expression by Western blot, and recombinant clones expressing LBPs are expanded, assayed for LDL binding and used to test compounds for their ability to inhibit LDL binding.

Example 18: In Vivo Screening for Agents Which Inhibit Binding Between LDL and LBPs

This example illustrates in vivo screening of agents which have been shown by in vitro tests to be promising candidate inhibitors of binding between LDL and LBPs.

In vivo inhibitory activity is first tested in the healing balloon-catheter deendothelialized rabbit aorta model of arterial injury (Roberts et al., J. Lipid Res. 24:1160-1167 (1983); Chang et al., Arterioscler. Thromb. 12:1088-1098 (1992)). This model was shown to be an excellent analog for human atherosclerotic lesions. Other useful animal models for human atherosclerosis include Apo E knockout mice and LDL receptor knockout mice. Both of these mouse models are characterized by high levels of plasma cholesterol and the development of naturally-occurring atherosclerotic-like lesions.

Each candidate inhibitor is tested in five to ten ballooned rabbits, while an equal number of rabbits receive a control peptide, or placebo. Four weeks following aortic deendothelialization, when reendothelialization (healing) is partially complete, daily parenteral (intravenous or subcutaneous) or intragastric administration of the peptides and the analogs begins at an initial concentration of 10 mg/kg body weight, which is varied down, or up to 100 mg/kg depending on results. 30 min later, a bolus of intravenously injected ^{125}I (or ^{99m}Tc-) labeled LDL is given to test the candidate inhibitor's ability in short term studies to inhibit LDL sequestration in healing arterial lesions. If ^{125}I-LDL is used, the animals are sacrificed 8-24 hr later, the aortas excised, washed and subjected to quantitative autoradiography of excised aortas, as previously described (Roberts et al., J. Lipid Res. 24:1160-1167 (1983); Chang et al., Arterioscler. Thromb. 12:1088-1098 (1992)). If ^{99m}Tc-LDL is used, analysis is by external gamma camera imaging of the live anesthetized animal at 2-24 hr, as previously described (Lees and Lees, Syndromes of Atherosclerosis, in Fuster, ed., Futura Publishing Co., Armonk, NY, pp. 385-401 (1996)),

followed by sacrifice, excision and imaging of the excised aorta. Immediately before the end of testing, the animals have standard toxicity tests, including CBC, liver enzymes, and urinalysis.

The compounds which are most effective and least toxic are then tested in short term studies of rabbits fed a 2% cholesterol diet (Schwenke and Carew, Arteriosclerosis 9:895-907 (1989)). Each candidate inhibitor is tested in five to ten rabbits, while an equal number of rabbits receive a control peptide, or placebo. Animals receive one or more doses per day of the candidate inhibitor, or placebo, for up to two weeks. Daily frequency of doses is determined by route of administration. If active drug or placebo are administered parenterally, they are given 1-3 times daily and the 2% cholesterol diet is continued. If drug or placebo are given orally, they are mixed with the 2% cholesterol diet. Schwenke and Carew (Arteriosclerosis 9:895-907 (1989)) have shown that the LDL concentration in lesion-prone areas of the rabbit aorta is increased 22-fold above normal in rabbits fed a 2% cholesterol diet for 16 days, and that the increased LDL content precedes the histological evidence of early atherosclerosis. Therefore, analysis of the effect of the candidate inhibitors is tested two weeks after the start of cholesterol feeding by injecting ^{125}I-LDL, allowing it to circulate for 8-24 hr, and then performing quantitative autoradiography on the excised aortas of both test and control animals. If appropriate, quantitation of aortic cholesterol content is also carried out (Schwenke and Carew, Arteriosclerosis 9:895-907 (1989); Schwenke and Carew, Arteriosclerosis 9:908-918 (1989).

The above procedures identify the most promising candidate inhibitors, as well as the best route and frequency of their administration. Inhibitors so identified are then tested in long-term studies of cholesterol-fed rabbits. These tests are carried out in the same way as the short-term cholesterol feeding studies, except that inhibitor effectiveness is tested by injection of ^{125}I-LDL at longer intervals following the initiation of cholesterol feeding, and lesion-prone areas of the aorta are examined histologically for evidence of atherosclerosis. Testing times are at two, four, and six months. Major arteries are examined grossly and histologically or evidence and extent of atherosclerosis. If necessary, other accepted animal models, such as atherosclerosis-susceptible primates (Williams et al., Arterioscler. Thromb. Vast. Biol. 15:827-836 (1995)), genetically altered

mice, and/or Watanabe rabbits are tested with short- and long-term cholesterol feeding.

Example 19: In Vivo Inhibition of Radiolabeled LDL Accumulation in the Ballooned Deendothelialized Rabbit Aorta via Induction of Active Immunity Against LBP Protein

This example illustrates the effect that induction of immunity against LBP protein has on the accumulation of radiolabeled LDL in the ballooned deendothelialized rabbit aorta model of atherosclerosis.

Immunity was induced in male New Zealand White rabbits (Hazelton Research Products, Denver, PA) as follows: A mixture of purified human recombinant LBP-2 or BHF-1 peptide (1 ml; 1 mg) and RIBI adjuvant (RIBI ImmunoChem Research, Inc., Hamilton, MT) was injected subcutaneously at 2-5 sites along the dorsal thoracic and abdominal regions of the rabbits. Blood was collected by venipuncture on days 1 (preimmune bleeding), 35, 63, and 91. Booster injections were administered on days 28 (500 μg; SC), 56 (250 μg; SC), and 84 (125 μg; SC).

The titer of the rabbits was evaluated by serial dilution using an ELISA plate format. Preimmune serum was evaluated at the same time. After the third booster of LBP protein or peptide, the titer reached a maximal level with a detectable calorimetric response on an ELISA plate of 156 pg. Titer is defined as the maximum dilution of antibody which generates an absorbance reading of 0.5 above control in 30 min. Specificity of the polyclonal antibodies was demonstrated using Western blot analysis as described in Example 6.

On day 93, the abdominal aorta of immunized and control rabbits was deendothelialized using a Fogarty number 4 embolectomy catheter (Chang et al., Arteriosclerosis and Thrombosis 12:1088-1098 (1992)). Four weeks after ballooning, rabbits received a bolus injection of ^{125}I-labeled LDL (1 ml; i.v.). Blood samples were collected at 1 hr intervals for 8 hr, and 24 hr post injection. Blood samples were centrifuged for 30 min at 2000 rpm (40°C)and total activity present in the serum was determined using a Gamma counter. Total TCA precipitable counts were determined by addition of TCA to the serum to a final concentration of 10% followed by incubation for 10 min at 4°C. Serum samples were then centrifuged (2000 rpm; 30 min; 40°C) and total

activity present in the supernate was determined. TCA precipitable counts were calculated by substration: total soluble counts minus counts present in the supernate after TCA precipitation. Blood samples for the determination of antibody titers were collected prior to the injection of the radiolabeled LDL.

After 24 hr, the rabbits were injected intravenously with 5% Evan's blue dye which was allowed to circulate for 15 min. Areas of the aorta in which the endothelial covering is absent stain blue while those areas covered by endothelium remain unstained. At the end of the incubation period, the rabbits were euthanized and the abdominal and thoracic aorta were dissected out, rinsed, and fixed overnight in 10% TCA at room temperature. The aortas were then rinsed exhaustively with physiological saline, weighed, counted, blotted dry and placed onto X-ray film in order to visualize the pattern of radiolabeled LDL accumulation in the deendothelialized rabbit abdominal aorta.

Immunization of rabbits against recombinant human LBP-2 or BHF-1 peptide altered the pattern of radiolabeled LDL accumulation in the ballooned deendothelialized abdominal aorta. When corrected for dosage, and percent reendothelialization, immunized-ballooned rabbits had lower accumulation of radiolabeled LDL compared to nonimmune-ballooned rabbits. These results indicate that active immunization against LBP provides an effective means by which the accumulation of LDL in the injured arterial wall can be modified.

Example 20: Screening Agents in Humans Which Inhibit Binding Between LDL and LBPs

Human studies are carried out according to standard FDA protocols for testing of new drugs for safety (Phase I), efficacy (Phase II), and efficacy compared to other treatments (Phase III). Subjects, who are enrolled into studies after giving informed consent, are between the ages of 18 and 70. Women who are pregnant, or likely to become pregnant, or subjects with diseases other than primary atherosclerosis, such as cancer, liver disease, or diabetes, are excluded. Subjects selected for study in FDA Phase II and Phase III trials have atherosclerotic disease previously documented by standard techniques, such as ultrasound and/or angiography, or are known to be at high risk of atherosclerosis by virtue of having at least one first degree relative with documented

atherosclerosis. Subjects themselves have normal or abnormal plasma lipids. Initial testing includes 20-50 subjects on active drug and 20-50 subjects, matched for age, sex, and atherosclerotic status, on placebo. The number of subjects is pre-determined by the number needed for statistical significance. Endpoints for inhibitor efficacy includes ultrasound measurements of carotid artery thickness in high risk subjects, as well as in subjects with known carotid or coronary disease; atherosclerotic events; atherosclerotic deaths; and all-cause deaths in all subjects. Non-invasive analysis (carotid artery thickness by ultrasound) as per Stadler (Med. and Biol. 22:25-34 (1996)) are carried out at 6- to 12-month intervals for 3 years. Atherosclerotic events and deaths, as well as all-cause deaths are tabulated at 3 years.

Oral dosage of drug in FDA Phase I trials ranges from 0.01 to 10 gm/day, and is determined by results of animal studies, extrapolated on a per kg basis. Based on data obtained from Phase I studies, the dose range and frequency are narrowed in Phase II and III trials. If parenteral administration of drug is determined by animal studies to be the only effective method, parenteral administration in human subjects is tested by injection, as well as by the transdermal and nasal insufflation routes. Testing of parenteral drug follows the same outline as that for oral administration.

The optimal treatment schedule and dosage for humans is thus established.

Example 21: Treating an Individual Having Atherosclerosis with BHF-1

This example illustrates a method for treating an individual having atherosclerosis with an LBP fragment, e.g., BHF-1, so as to decrease the levels of arterially bound LDL in the individual. BHF-1 is obtained as described herein. The BHF-1 is administered to the mammal intravenously as a bolus or as an injection at a concentration of 0.5-10 mg/kg body weight. Such administrations are repeated indefinitely in order to prevent the development or progression of symptomatic atherosclerosis, just as is done currently with cholesterol lowering drugs. Stable subjects are examined twice yearly to evaluate the extent of any atherosclerotic disease by physical exam and non-invasive studies, such as carotid artery thickness, ultrasound, and/or gamma camera imaging of the major arteries, to determine if atherosclerotic lesions are present, and, if previously present, have regressed or progressed. Such a regimen results in treatment of the atherosclerosis.

Example 22: In Vivo Reduction of Atherosclerosis in Apo E Knockout Mice by Immunization with LBPs

Separate immunization experiments were performed with each of LBP-1, LBP-2, and LBP-3. Immunity was induced by injecting apo E knockout mice with the LBP protein (LBP-1, LBP-2, or LBP-3) together with an RIBI adjuvant (RIBI ImmunoChem Research, Inc., Hamilton, MT). Apo E knockout mice (Jackson Laboratories, Bar Harbor, ME) are hyperlipidemic and thus a model for human atherosclerosis. Apo E knockout mice have high levels of plasma cholesterol and develop naturally-occurring atherosclerotic-like lesions.

Four week old apo E knockout mice (Jackson Laboratories, Bar Harbor, Maine) were ear tagged, randomly assigned to different cages and weighed. Body weights were determined weekly. Animals were allowed to habituate for 1 week. Normal rodent chow was provided *ad libitum* and animals were maintained in a 12:12 light:dark cycle. The following four groups of mice were treated with either recombinant LBP proteins (40 μg of recombinant protein/mouse) plus RIBI adjuvant or RIBI adjuvant alone (control group).

LBP-1: Immunized with rabbit recombinant LBP-1 (6-His tag).
LBP-2: Immunized with rabbit recombinant LBP-2 clone 26 (6-His tag).
LBP-3: Immunized with rabbit recombinant LBP-3 (6-His tag).
Control: Received adjuvant.

Blood samples (pre-immune serum) were collected prior to the initial injection of recombinant protein and RIBI adjuvant (as described in the manufacturer's manual). After 21 days, mice received a booster injection (half-initial dose) and were then bled seven days later. Titer was defined as the maximum dilution of serum that yielded a change in absorbance equivalent to 2X that of control serum (60 min; 37°C). The amount of recombinant protein per well was 100 ng.

Booster injections took place at 21 day intervals until an average titer value of 1:10,000 was reached. At this time, mice were switched to western type diet (Harland

Teklad, Madison, WI) and fed *ad libitum*. Blood samples were collected at this time (retro-orbital sinus bleeding technique) and monthly thereafter.

Blood samples were analyzed for total cholesterol, HDL cholesterol, and triglyceride concentration with a commercially available total cholesterol and triglycerides assay kits (Sigma; St. Louis MO) using an ELISA format. HDL concentration was determined after Apo B containing lipoproteins were precipitated using heparin/$MnCl_2$.

Apo E knockout mice were sacrificed at 26 weeks of age. The mice were anesthetized with methoxyfluorane and exanguinated via cardiac puncture. A midline thoracotomy was performed, a cannula inserted into the right ventricle and perfusate allowed unrestricted flow via an incision into the right atrium. The mice were perfused with saline, followed by 10% phosphate buffered formalin until fasciculations stopped. At this time, the aorta was exposed and adventitial fat removed *in situ*. The aorta was then removed from the heart down to the iliac bifurcation and placed in 10% phosphate buffered formalin overnight.

The aorta was stained as follows: after a brief 70% ethanol rinse, it was immersed in a filtered solution of 0.5% (weight/volume) Sudan IV in 35% ethanol/50% acetone with continuous shaking for 10 minutes at room temperature. Unbound dye was removed by incubating the aorta in an 80% ethanol solution with shaking until the background color was clear. The vessel was then rinsed in distilled water, placed in physiological saline and opened longitudinally from the aortic arch down to the iliac bifurcation. The vessel was pinned out and photographed. Photographs were then digitized using an Astra 1200S scanner (UMAX Technologies Inc., Freemont, CA) and a commercially available graphics program (Canvas; Deneba Software, Miami Fl). Total and lesion areas were determined using the signal processing toolbox of MATLAB (The Mathworks Inc., Natick, MA). Percent involvement was calculated by dividing lesion area by total area.

A second analysis was done to measure aortic atherosclerosis by a cholesterol extraction method whereby cholesterol is determined as a unit weight of artery. This method may be more accurate in measuring lesion size than attempting to measure the thickness of many sections. Specifically, the weight of an artery was measured, then the cholesterol was extracted. Aortic cholesterol content was then measured by gas-liquid

chromatography. The amount of cholesterol per unit weight of artery was then determined.

After the first booster injection, some of the apo E knockout mice immunized against LBP-1 had relatively high anti-LBP-1 titers (≤1:5000) while others in the same group exhibited moderate levels (>1:500 to <1:1000). LBP-2/26 titers were low in the apoE knockout mice (<1:500) at this time. LBP-3 titers ranged from moderate to low (≥ 1:500 to < 1:1000) to low (<1:500) in the apoE knockout mice.

After the second booster injection, Apo E knockout mice immunized against LBP-1 had moderate to high titers (>1:1000 to ≤ 1:8000). Apo E knockout mice immunized against LBP-2/26 had moderate titer levels (>1:2000). LBP-3 titers range from moderate to high (>1:1000 to > 1:8000) in the Apo knockout mice.

After the third booster injection, most of the mice immunized against LBP-1 had relatively high titers (>1:10,000) while others had moderate to high titers (>1000 to < 1:10,000). Some of the Apo E knockout mice had moderate (<1:5000) to low (<1:1000) titers. LBP-3 titers ranged from high (>1:5000 to ≤ 1:10,000) to moderate (>1:1000 to <1:5000).

Data were analyzed using T-tests and Wilcoxons. Immunization against LBP-1, LBP-2/26 or LBP-3 did not have a significant effect ($P > 0.05$) on body weight of Apo E knockout mice. Due to the small sample size and the large variability present in the Apo E knockout mice, it was not possible to determine whether immunization against LBP-1, LBP-2/26 or LBP-3 had an effect on total cholesterol, HDL cholesterol or triglycerides concentration, but it did not appear to.

Immunization against LBP-1 or LBP-3 did not have a significant effect ($P > 0.05$) on lesions of the apo E knockout mice or LDL receptor negative knockout mice. However, immunization of the apo E knockout mice against LBP-2 had a significant effect on lesion area (Table 2), and, once outliers were deleted, a significant effect on arterial wall cholesterol content (Table 3). The LBP-2 immunized apo E knockout mice had significantly reduced aortic atherosclerosis as compared to the control, non-immunized mice. Without being bound to any particular theory, the circulating antibodies generated against LBP-2 proteins are thought to block LDL binding to the artery wall.

Table 2 – Lesion Area in LBP-Immunized Apo E Mice

Apo E Mice	Lesion Area % Coverage	Treated Area Change	P-Value Wilcoxon
Control	9.40		
LBP-1	6.05	-0.36%	0.07
LBP-2	6.01	-0.36%	0.01
LBP-3	7.14	-0.24%	0.36

Table 3 – Arterial Cholesterol Content in LBP-Immunized Apo E Mice

Apo E Mice	Arterial Wall Cholesterol (ug cholesterol/mg aorta)	Treated Area Cholesterol Change	P-Value Wilcoxon
Control	6.33		
LBP-1	3.82	-0.40%	0.14
LBP-2	3.28	-0.48%	0.07
LBP-2 (outliers deleted)	1.83	-0.71%	0.01
LBP-3	4.48	-0.29%	0.20

Example 23: In Vivo Reduction of Atherosclerosis in LDL Receptor Knockout Mice by Immunization with BHF-1

An immunization experiment was performed with the BHF-1 peptide. LDL receptor (LDLR) knockout mice (B6,129S-Ldlr^{tm1Her}, Jackson Laboratories, Bar Harbor, ME) were injected with the BHF-1 peptide (see Example 15 for methods of synthesizing the BHF-1 peptide) together with an RIBI adjuvant (RIBI ImmunoChem Research, Inc., Hamilton, MT). LDLR knockout mice are hyperlipidemic and thus a model for human atherosclerosis. LDLR knockout mice have high levels of plasma cholesterol and develop naturally-occurring atherosclerotic-like lesions.

Four week old LDLR knockout mice were ear tagged, randomly assigned to different cages and weighed. Body weights were subsequently determined weekly. Animals were allowed to habituate for one week prior to experimentation. Normal rodent chow was provided *ad libitum* and animals were maintained in a 12:12 light:dark cycle. Animals were divided into experimental and control groups, as follows: (1) experimental, 16 mice were immunized with the BHF-1.20.L peptide; (2) control, 8 mice were immunized against bovine serum albumin.

Mice in the experimental group received subcutaneous injections (9.99 μg/g body weight; 200 μl final volume) of the BHF-1.20.L peptide daily for 2 weeks, from 5 to 7 weeks of age, prior to the initial injection with the peptide and adjuvant. Blood samples (pre-immune serum) were collected prior to the initial injection of BHF-1.20.L and RIBI adjuvant (50 μg of peptide/mouse) (as described in the manufacturer's manual) at 7 weeks of age. After 21 days, mice received a booster injection (half-initial dose) and were then bled 7 days later. Titer was defined as the maximum dilution of serum that yielded a change in absorbance equivalent to 2X that of control serum (60 min; 37°C). The amount of peptide per well was 100 ng. Booster injections took place at 21 days interval.

Blood samples were analyzed for total cholesterol, HDL cholesterol, and triglyceride concentration, using commercially available total cholesterol and triglycerides assay kits (Sigma; St. Louis MO) (ELISA). HDL concentration was determined after Apo B containing lipoproteins were precipitated using heparin/$MnCl_2$.

When fed a normal rodent chow, total serum cholesterol concentration in LDLR knockout mice remains relatively low. A high fat diet, on the other hand, results in an increase in total serum cholesterol concentration in these mice. The animals were thus switched at 16 weeks of age to a modified "Western Type" diet (0.1% cholesterol

content) (Harland Teklad, Madison, WI) and fed *ad libitum*. This diet was expected to increase the total serum cholesterol concentration to a range of 600-800 mg/dl, thereby increasing the rate of lesion formation. Blood samples were collected at 18 weeks of age (retro-orbital sinus bleeding technique) and monthly thereafter.

At 30 weeks of age, the mice were sacrificed and aorta were removed as described in Example 22. Aortic atherosclerosis was measured by the cholesterol extraction method described in Example 22, whereby cholesterol is determined as a unit weight of artery.

Immunization against BHF-1.20.L had no effect on body weight of LDLR knockout mice. Consumption of the modified "Western Type" diet for 12 weeks significantly ($P<.05$) increased total serum cholesterol, HDL cholesterol and triglycerides in both experimental and control animals. Levels of total serum cholesterol, HDL serum cholesterol and serum triglyceride concentration were not significantly different ($P>.05$) between experimental and control animals.

Mice that were immunized with the BHF-1 peptide had 24% less aortic cholesterol content ($P>0.037$) as compared to the control, non-immunized mice. Without being bound to any particular theory, the immunization is thought to generate circulating antibodies against the BHF-1 peptide. These antibodies are thought to block LDL binding to the artery wall, thereby reducing aortic cholesterol content.

Those skilled in the art will be able to ascertain using no more than routine experimentation, many equivalents of the specific embodiments of the invention described herein. These and all other equivalents are intended to be encompassed by the following claims.